

What price does Yeltsin command?

The case for providing financial assistance to the Russian government is still strong, even if President Boris Yeltsin is less secure now than he was a year ago.

PITYING poor Russia is now popular again. For some days, President Bill Clinton has been dropping hints that he, unlike his predecessor, is creditably willing to contemplate substantial help for Mr Boris Yeltsin's government, still locked in combat with the Congress of People's Deputies on the question "Who runs Russia?". At the weekend, the finance ministers of the seven major industrial nations heard at a meeting in Tokyo of Russia's present financial needs. Or, rather, they will have been told of the needs the Russian government believes they may be prepared to satisfy. The true need is, on present form, probably indistinguishable from infinity.

To say that effective assistance for Russia and the other republics of the Soviet Union would have been easier to provide six months or more ago, before inflation had further eaten into people's resources and before Yeltsin's authority had been undermined by the chaotic events of the past few months, is true but beside the point. Russia and the rest of us are where we are now. And the issues that divide Yeltsin and his government from the Congress are no longer simply those of the price it is worth paying for economic reform, but also involve the degree of trust that each has in the other. So many harsh things have been said in the past few months, and especially since December, that there can be very little left.

In the circumstances, it is surprising that Russia still hangs recognizably together. It is not merely that the excellent Moscow metro continues to function, but that many of the better laboratories continue much as they were a few years ago, even if their ranks are thinned by the absence of able people — temporary, permanent but often unknown — and the departure of others for activities that offer more immediate rewards, especially in what is called the market. As always when the authority of government collapses, there have emerged networks of people with common interests who are able to do much to help each other. The social tragedy is that there is very little that those united only by their dependence on a state pension, or by their membership of the armed services, can do to help themselves.

This cannot continue. This is not the first time since Mr Mikhail Gorbachev took office that there has been talk of civil war. Mercifully, for all the bitterness of the recent conflict between the Congress and the government, there is no evidence that some faction in the Congress will seek power from the government by force. Nor will Yeltsin use force to bring the Congress to heel; he will know that that would put paid to any chance of outside help. So is he worth the gamble of

substantial outside help, say \$25 billion or thereabouts? Damaged though he is, Yeltsin is the only custodian of such funds in sight, and there is a chance that they might help him get a grip on a daunting array of problems. But it is a gamble, and one that will distract those who provide the funds from helping the other countries of Eastern and Central Europe, which are even more deserving. □

Manufacturing doubts

The impending reorganization of British research should be coloured by the causes of industrial decline.

THE British government's plan to produce a policy statement on research later in the summer was never going to be easy. To the extent that one of its purposes is to bend the energies of the research community to the solemn (and necessary) business of 'wealth-creation', there has been a danger from the outset that researchers would be obliged to search more assiduously for industrially applicable innovations that would then fail to capture the interest of British industry. That suspicion has now been strengthened by the leaking, last weekend, of the contents of an internal report by officials of the British Department of Trade and Industry. Its conclusion is that the relative failure of British industry over many years are intrinsic to itself.

Technical people in Britain will not be surprised by that conclusion, but the mere existence of the study (commissioned by the Secretary of State for Trade and Industry, Mr Michael Heseltine) shows a refreshing willingness to be self-critical. By all accounts, the group of civil servants responsible has visited Britain's industrial competitors, in Europe and North America, which is in itself a sign of grace. Among its findings are that the price advantage conferred on British manufacturers by the depreciation of sterling (down more than 15 per cent since last October's expulsion from the European Exchange Rate Mechanism) will not, in general, compensate for the lack of innovation embodied in the products manufacturers want to sell. The group believes that a long time must pass before this can be remedied.

Remarkably, Heseltine appears to accept this analysis. He has no choice. Speaking last weekend, he claimed that the decline of British industry can be traced back nearly a century to the social separation between manufac-



turers and the rest of society, the governing classes in particular. He agrees that this state of affairs will not be quickly cured. But, shamefully, he is not sure whether the report will ever be published, saying that it would identify for competitors the weak points in British industry, as if they did not know already.

Economic statistics point to one disturbing structural weakness in the British economy: at times of economic growth, the balance of external payments for goods traded internationally tends to go awry. In other words, British industry cannot satisfy the demands of its domestic market when people have money in their pockets. If and when the present recession ends, that effect promises an old-fashioned balance-of-payments crisis whose severity will be the first objective measure of the degree to which the decline of British industrial capacity has been further exacerbated by the recession. The continuing announcements from large companies of their intentions to make workers redundant hint at what is happening. Corporate bankruptcies have been running at more than 60,000 a year for some years.

It is more disturbing that very similar diagnoses of Britain's economic weakness have been produced regularly for the best part of the past half-century. Immediately after the Second World War, several working groups of the Anglo-American Productivity Council pointed to the scant attention paid to innovation and the employment of innovators in British industry. In 1957, the British Association for the Advancement of Science (whose serious influence has itself since declined) produced an influential document saying much the same. With a few exceptions (pharmaceuticals now), British industry seems to have lost both the flair and the appetite for major technical innovations.

So much is clearly witnessed by the decision of the London Stock Exchange last week that it will abandon a software project for the automatic settlement of stock sales and purchases on which it has already spent £75 million, perhaps a third of total spending on the project. Although the Thatcher governments seemed often to believe that the decline of manufacturing would be immaterial if there were a compensating growth in the provision of services, there can hardly be a more vivid proof that, these days, even the sale of services requires technical competence of a high order.

The bearing of all this on the planned white paper on research is obvious. Crude attempts to force applicable innovations out of the research community may harm research but, in themselves, do nothing for prosperity. What good is a technical innovation that, when patented or otherwise protected, is not fully exploited? That is why an effective policy for research directed at wealth-creation must hang on more successful attempts than there have been so far to make manufacturing companies more technically skilled and competent — more aware of their dependence on skilled people for their success. But that, of course, lies outside the remit of the minister responsible, Mr William Waldegrave. But Heseltine's leaked document should at least give him ammunition with which to fend off simplistic demands from Heseltine's department. □

Angry summer time

Britain is out of step with the rest of Europe even on matters such as summer time. But that may change.

WITH summer approaching, the issue of British daylight saving has once again appeared above the horizon, seemingly bent on pursuing its annual ascent through the political firmament. At a conference last month, the vociferous Daylight Extra Action Group renewed its call for Britain to get into step with the rest of Europe by setting its clocks to Central European Time (CET), an hour ahead of Greenwich Mean Time (GMT), Britain's winter standard which is adjusted every summer by one hour (as in the rest of Europe). But despite the usual grumblings, the well-worn arguments about whether it is more dangerous for a child to walk home from school in the dark than it is for a farmer to operate a combine harvester without proper visibility, the opposition to synchronization seems to have all but caved in.

The British government has traditionally tried to minimize its involvement with this surprisingly emotive debate. In 1989, it issued a consultation document outlining the options as it saw them. Britain could make a wholesale transition to CET, the so-called 'single-double summertime' option, involving a two-hour leap forward one spring, and a step back of an hour the same autumn; it could go half-way, and synchronize the end-date of Britain's summer with the rest of Europe (at present there is about a month's discrepancy); or it could leave things exactly as they are.

Feedback from the public was conflicting. The more that rural northern Britain was in favour of preserving the status quo, the more the south called for CET. The whole issue, in short, became politically rather sensitive. Relief came, in the end, from an unexpected quarter. A review by minister Peter Lloyd after the Conservatives' surprise general-election victory last year revealed a movement gaining momentum among Eurocrats in Brussels to abolish summer time altogether. The French particularly, whose agricultural lobby makes daily headlines by its restlessness in the free global marketplace, appear to be in favour of this Scroogeish sounding proposal, providing the British government, until the issue is settled, with the perfect excuse for keeping the issue on a back-burner.

But domestic pressure may yet force the government's hand. According to Angus Creighton-Miller of the Daylight Extra Action Group, the National Farmers' Union has assured him that its members are "pretty agnostic" these days about daylight saving, and are unlikely to lobby against any move that results in an extra hour of darkness every morning. Those in favour of the switch, by contrast, have never been more outspoken. Last month's conference called for a quick switch to Central European Time "so that lives can be saved and the quality of life be enhanced for all". The government may well listen if it wins the Maastricht Bill it is fighting through the House of Commons, but otherwise may not have the stomach for even this modest European venture. □

Research spending is almost flat for leading Japanese companies

Tokyo. The global recession that reached Japan's shores last year is beginning to bite harder into research and development (R&D) spending by Japan's major industries. A *Nature* survey of 14 leading electronics and automobile manufacturers that account for 30 per cent of Japan's total industrial research reveals that total R&D expenditures for the fiscal year ending 31 March will rise barely above last year's level, ending a ten-year period of rapid expansion. In addition, several companies expect to reduce R&D spending in 1993 for the first time in several decades.

The shift, which has already led companies to recruit fewer young scientists and engineers, has also forced researchers to think more about product development. This restructuring of research goals is likely to be the most important long-term consequence of the recession.

The 14 companies surveyed spent a total of ¥3,178 billion (US\$27 billion) on R&D in 1991. Projections for fiscal year 1992 (ending 31 March) show an increase of only 1.5 per cent, with one company — Oki Electric Industry — in which R&D spending dropped from ¥44.4 billion in 1990 to ¥40.9 billion in 1991 declining to provide figures for 1992.

Prospects for the coming year appear to be no brighter, with several companies looking at flat or declining R&D budgets in the fiscal year that starts on 1 April. Hitachi, with the largest R&D budget of the companies surveyed, expects spending to fall by 4 per cent, from ¥520 billion to ¥500 billion. One of the smaller companies, Sanyo, forecasts a drop from ¥85 billion in 1992 to ¥61 billion in fiscal year 1993, which for Sanyo ends on 30 November, and Kyocera, the world's leading manufacturer of ceramic packages for integrated circuits, expects a decline of 17 per cent in fiscal 1993.

Toshiba expects its budget to be "about the same or slightly less" in 1993 after a drop of 2.5 per cent in 1992, while NEC expects its R&D budget to be flat. Two leading car manufacturers, Nissan and Mazda, spent slightly less on R&D in 1992 than in 1991.

A Toshiba researcher says that the situa-

tion "is not desperate" but that travel to conferences overseas has been "severely cut" and is restricted to reasons related to product development or patents. The company for a time switched off the lighting in

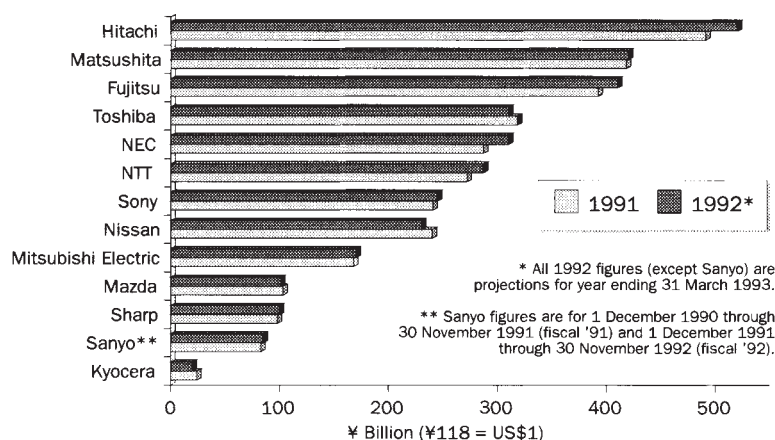
simply be offering management the chance to implement them.

Toshiba has consolidated its laboratories at an R&D centre in Kawasaki near Tokyo, with some to be reassigned to product development centres. Researchers also are being asked to siphon off ideas that could quickly lead to new products. Again, the recession seems to have provided an excuse to implement plans already in the works.

The changes seem less dramatic at NEC, although basic researchers are being encouraged to explain the potential long-term applications of their work and to transfer to product development centres any ideas that have immediate potential application. Furthermore, they are being asked to discuss with applied researchers the likely direction of future technologies.

The words "strategy and priority" are being used much more today than a few years ago when funds were plentiful, says the head of one of NEC's basic research laboratories. **David Swinbanks**

Rise in research spending stalls



Source: Individual company documents

its laboratories during the lunch period to drive home the message that sacrifices are necessary, although the surge in electricity when the lights go back on actually increased consumption. Nevertheless, it had an important "psychological effect" on researchers, who are looking more closely at proposals and trying wherever possible to cut costs.

Recruitment is also suffering, with Fujitsu announcing last week that it will recruit only 300 engineers in the coming fiscal year compared with 2,200 in 1992. NEC expects a drop of 10 per cent, from 990 to 900, and Hitachi expects overall recruitment, including high school graduates, to drop from 4,000 to 2,500.

More important than these cutbacks, which are likely to be temporary, are moves by top management to restructure the way in which research is carried out and funded. Before the recession, for example, the main corporate research laboratories at Sony were funded by headquarters. Now researchers are being asked to get a large percentage of their research budgets from the 24 business groups that make up Sony.

These business groups operate as independent companies and look for research that is product-orientated. As a result, Sony researchers will have to define more clearly the goals and nature of their work. Such plans were already afoot before the recession, and the freeze on R&D spending may

Babbitt moves ahead on biological survey

Washington. In the first step towards creating a new National Biological Survey to consolidate ecological research within his agency, Bruce Babbitt, US interior secretary, has asked the US National Academy of Sciences to advise him on how to organize the new survey (see *Nature* 361, 574; 1993).

Peter Raven, home secretary of the academy and director of the Missouri Botanical Garden in St Louis, will chair the committee, which is expected to complete its work within six months. Babbitt has asked biologist Thomas Lovejoy of the Smithsonian Institution to join the department on a temporary basis to set up the new survey, which would monitor and inventory species and ecosystems within the United States. The new office will have a budget of \$12 million, and Lovejoy will also serve as acting science adviser to Babbitt.

Tony Reichhardt

Amgen to spend \$80 million to create Canadian institute

Washington. A Canadian subsidiary of Amgen Inc. of Thousand Oaks, California, will spend C\$100 million (US\$80 million) over the next ten years to create a medical research institute in Toronto that will study the human immune system and how it relates to diseases such as cancer. The institute is another indication that the US biotechnology industry has matured to the point where its leading companies can begin to emulate the global pharmaceutical giants by looking outside their own laboratories for the next blockbuster drug. However, that strategy is not without its own risks (see below).

The Amgen Institute will be located next to and will retain strong links with the Ontario Cancer Institute/Princess Margaret Hospital (OCI/PMH). It will be headed by Tak Wah Mak, an immunologist who in 1984 discovered the T-cell receptor. Mak resigned as head of the division of cellular and molecular biology at OCI/PMH to take up the new post but will still be a member of that department. The affiliation agreement will allow OCI/PMH, Canada's largest cancer treatment and research centre which is affiliated with the University of Toronto, to create a fully endowed research chair and strengthen its training programme as well as to recruit top scientists to the area. The grant-funded research arm of OCI/PMH has an annual budget of about C\$15 million.

In return for its investment, Amgen has acquired the exclusive rights to all pending patents currently held by the cancer centre in the area of gene knockout mice (based on Mak's work for the past four years) and all rights to any inventions derived from the new institute. Those rights extend only to the work of Mak and employees of the new institute and not to the 36 senior scientists who will continue to work for the cancer centre. As part of the affiliation agreement, the cancer centre will retain some influence over the governance, recruitment and review procedures of the new institute.

Founded in 1980 and with two of the top-selling biopharmaceuticals on the market (in Epogen and Neupogen), Amgen is in the enviable position of being one of a few biotechnology companies generating product sales, with revenues last year of US\$1.128 billion. Setting up a complementary research arm with close ties to an academic institution will provide Amgen with "a direct pipeline into the newest technologies, techniques and information", says Mak.

Preliminary negotiations were focused on a collaboration with the cancer centre. Eventually, however, Amgen decided to create an institute affiliated closely with OCI/PMH and led by Mak that would carry

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Canada's Tak Mak is the man for Amgen.

out basic research. "In order to get a productive research unit you need a man at the top", says Daniel Billen, general manager of Amgen Canada. "That man at the top for us is Tak Mak."

The new institute, to be completed in March 1994, will occupy 25,000 square feet

in a building owned by OCI/PMH that will be adjacent to and physically joined by a connecting bridge to a new OCI/PMH facility to be ready the following year. When fully staffed, the institute will employ about 70 people, including a dozen or so senior research scientists.

Mak says that he will be looking for individuals with backgrounds in lymphocyte activation, neurobiology and the regulation of cell growth as well as specialists in immunoregulation, his own area of interest.

New employees will also be eligible for cross-appointments in the research divisions of the cancer centre and will be able to apply for research grants from outside sources.

Under the terms of the 10-year agreement, the cancer centre will receive C\$2 million from Amgen to fund a fully endowed research chair, most likely in molecular biology, as well as money to expand by 10 positions a year its current level of 60 graduate students and 50 postdoctoral scientists. The cancer centre will also receive payment by Amgen for use of its core facilities, including its animal colonies and media

preparation facilities.

Although this will be the first centre of its kind set up by Amgen outside its California headquarters, Billen says that the company has plans to fund two additional research centres — both in the United States.

Diane Gershon

Terms of Scripps-Sandoz agreement may be more common than its critics believe

Washington. Bernadine Healy, the outgoing director of the US National Institutes of Health (NIH), last week sharply criticized an offer from the Sandoz Pharmaceuticals Corporation of New Jersey to spend \$300 million over ten years at the Scripps Research Institute in La Jolla, California. In testimony that was not screened by her political bosses, Healy told a congressional subcommittee chaired by US Representative Ron Wyden (Democrat, Oregon) that the proposed contract "does not seem to be in the best interests of the biomedical community, the public or US competitiveness" and that it "does not match similar agreements between other nonprofit laboratories and industry".

The first point is open to interpretation, of course; Wyden is concerned that Sandoz will have a monopoly over \$100 million a year in federal research in return for its investment, a view hotly disputed by officials from Scripps and Sandoz. An answer to the second point must await the responses of 104 academic institutions to a request

made last month by NIH to describe their collaborations with industry. But a preliminary analysis suggests that Healy's description of the agreement as an "aberration" is, in certain respects, incorrect.

At the heart of the Scripps-Sandoz agreement, due to take effect in 1997, is a panel of seven scientists each from Scripps and Sandoz that would decide how to spend \$12 million of the \$30 million a year coming from Sandoz. (The rest will go into a general account for nondirected research.) The group, called a Joint Scientific Council, would operate much like an NIH study section, rating proposals submitted by Scripps researchers and funding those it likes best.

That is precisely the way an advisory committee created by the largest existing collaboration between a pharmaceutical company and a US research institution — a \$100-million agreement between Monsanto and Washington University medical school in St Louis, Missouri — has operated since 1982. Once a year, five senior scientists from each institution sift through the re-

M.A. Woodford

sponses to a request for proposals and choose those most worthy of support.

Last year, for example, the Monsanto–Washington University committee funded about a third of the proposals it received; at any given time, the \$9 million a year from Monsanto is supporting about 50 projects. Those rejected are free to find other support, typically from NIH; the same would be true at Scripps. The concurrence rate between university and company scientists “is very high”, says David Kipnis, who helped to negotiate the agreement and who was until recently chairman of the department of medicine at the medical school.

No such council exists in the current agreement between Scripps and Johnson & Johnson (J&J) of New Jersey, on which the company spent \$10.5 million last year. (As in the Sandoz agreement, J&J spends some of its money on specific projects with the rest going into a general pot for use as Scripps sees fit.) Instead, there is a medical utility evaluation committee that discusses the results of work by Scripps scientists; anyone doing work deemed of interest to the company is asked to submit a proposal, which is reviewed by several J&J officials. “We think that the council is a much better way of doing things”, says William Beers, a senior vice president at Scripps. “It’s something that scientists are already used to.”

Healy last week testified that the agreement “forbids too many things and runs counter to the spirit of science”. In particular, she is concerned about the authority of Sandoz officials to terminate research projects that they feel are ripe for development and to review existing agreements between Scripps researchers and other companies as they expire.

Beers admits that at present J&J “does not have the right to intercede” on the fate of existing agreements with other companies but that under the Sandoz contract “we will have to go to them [Scripps] first”. However, he says that Sandoz would not assume control of a project until it is ready for commercialization and that Scripps “is not in the business of developing drugs”. Speaking only about his university’s collaboration with Monsanto, Kipnis says that “most of the time it’s the other way around: a scientist thinks that he has a cure for something and wants it to be developed right away and the company says it’s not interested.”

Lawyers for Scripps believe that Healy has spent insufficient time reading a lengthy technical document and that her comments misinterpret the spirit of the contract. Healy acknowledges that NIH lawyers had barely a day to review the agreement, which may also explain why her sharpest criticism did not appear in the advance version of her testimony that is routinely reviewed by White House officials. The Clinton administration has no official position on the controversy, which arose shortly after the agreement was announced in early December.

Jeffrey Mervis

British JET workers protest against hiring discrimination

Oxford. Fusion research at the Joint European Torus (JET) laboratory at Culham in Oxfordshire continues to be disrupted by British scientists striking in protest against future employment barriers based on age and citizenship.

their colleagues are employed by EC’s nuclear agency, Euratom. As a result, British researchers are treated as external applicants when applying for positions at other EC projects.

Some 150 workers participated in the most recent strike, held on 5 March. It was a response to the rejection on 26 February by the JET Council of their request to be made temporary EC employees. Last September, an independent panel appointed by Filippo Pandolfi, then EC research commissioner, recommended that the AEA staff at JET should be given temporary EC contracts or at the least that they should in future receive preferential treatment over outside applicants. The JET Council rejected



Striking JET workers and their families offer their views.

JET is due to close at the end of 1996, and many of its 420 employees hope to be employed by other research projects supported by the European Commission (EC). That has already happened to 13 of the 20 staff made redundant last year. But the remaining seven — all Britons — have failed to find work partly because of an EC policy that new employees must be under 35 years old for junior posts and under 50 for senior posts.

British workers at JET are employed by the UK Atomic Energy Authority, while

the first suggestion and endorsed the second, which it has no power to implement.

The only concession so far to British workers has been to raise the age limit to 50 for all vacancies at the forthcoming International Thermonuclear Experimental Reactor (ITER) project. But representatives of the striking workers say that the change is too little, too late: ITER is only one of many EC projects, and it will employ only a hundred people in its initial stage.

Oliver Tickell

NASA shrinks station, elevates life sciences

Washington. Facing a mismatch between money and ambition, US President Bill Clinton has told the National Aeronautics and Space Administration (NASA) to design a smaller space station with fewer functions than the one now being built. The new station will orbit for half the scheduled 30 years, will be easier to maintain and may not be permanently inhabited. Some 40–50 engineers and scientists from NASA, industry, universities and the project’s partners in Europe, Japan and Canada have been given until 1 June to propose options.

The overall cost to build the new station will be “significantly less” than the former estimate of \$31 billion, according to NASA Administrator Daniel Goldin. Lifetime operational costs for the facility — which have been estimated to be as high as \$100 billion — will be reduced by half. NASA has al-

ready spent \$8.5 billion on the project, and the redesign team, will try to retain as much of that work as possible. Clinton wants the station to be completed within five years and he wants NASA to begin conducting research — whether on the space station, on a space shuttle modified for long stays in orbit or on a Russian orbiting station — by 1997.

Last week, on the same day as Goldin announced the station redesign, NASA also announced changes in the organization of its research programme. New offices within the agency will be responsible for life and microgravity sciences; advanced concepts and technology; planetary science and astrophysics; and Earth science. The reorganization is consistent with NASA’s emphasis on biological research as an essential activity for the station.

Tony Reichhardt

New programme of European fellowships wreaks havoc with local pay scales

Munich. A new programme of postdoctoral fellowships has caused a financial and political upheaval throughout Europe, with salaries varying as much as fourfold and researchers criticizing the organizing body for failing to make the rules clear.

Four months after the first fellowship grants from the European Commission's (EC) Human Capital and Mobility programme, participating countries are still trying to work out how to pay their fellows. Faulty assumptions about the appropriate size of the stipend have made researchers into either princes or paupers and created disruptive inequalities within laboratories.

The ECU488 million (US\$710 million) programme, which offers fellowships of up to two years to work in another European country, was designed to foster collaborations across national boundaries. But it has had problems since its inception last summer. The original announcement set a deadline of only two to three weeks, not nearly enough for the necessary coordination between countries (see *Nature* 359, 178; 1992). Similarly, when the first grants were awarded in November, universities found that it was impossible to reconcile them within their own laws.

The fellowships provide the host institution with a lump sum to pass on to the individual, but the amount was chosen without consulting the institutions that would be handling the grant. Twelve per cent is allowed for administration and overhead, a fraction so small that some universities are reconsidering their decision to participate.

None of the money is for bench costs, a point widely criticized by scientists. Jörg Schneider, a German coordinator of EC programmes, believes that this prohibition, combined with severe overcrowding at most universities, has discouraged German scientists from agreeing to host fellowships. As a result, only 82 German institutes applied as hosts, compared with 316 in Britain and 237 in France.

The fellowship is supposed to go entirely to the researcher, less an undefined small amount for the cost of publications and conferences. But the guidelines do not specify whether the stipend is a grant and, therefore, not subject to tax and social security payments or a normal salary, in which net income would vary considerably between countries according to national tax and social security laws.

Administering the payments has proved

to be a nightmare. For the most part, host institutes have to negotiate individually with each fellow. "Every single thing that has come out of the programme has been designed to make life more difficult, not easier", says Charlotte Beatson of the University of Oxford. Countries that have left the Exchange Rate Mechanism, such as Britain and Spain, must also deal with the unpredictable fluctuations in exchange rates between local currency and the ECU. In many countries, some money is being held back pending an acceptable system of payment.

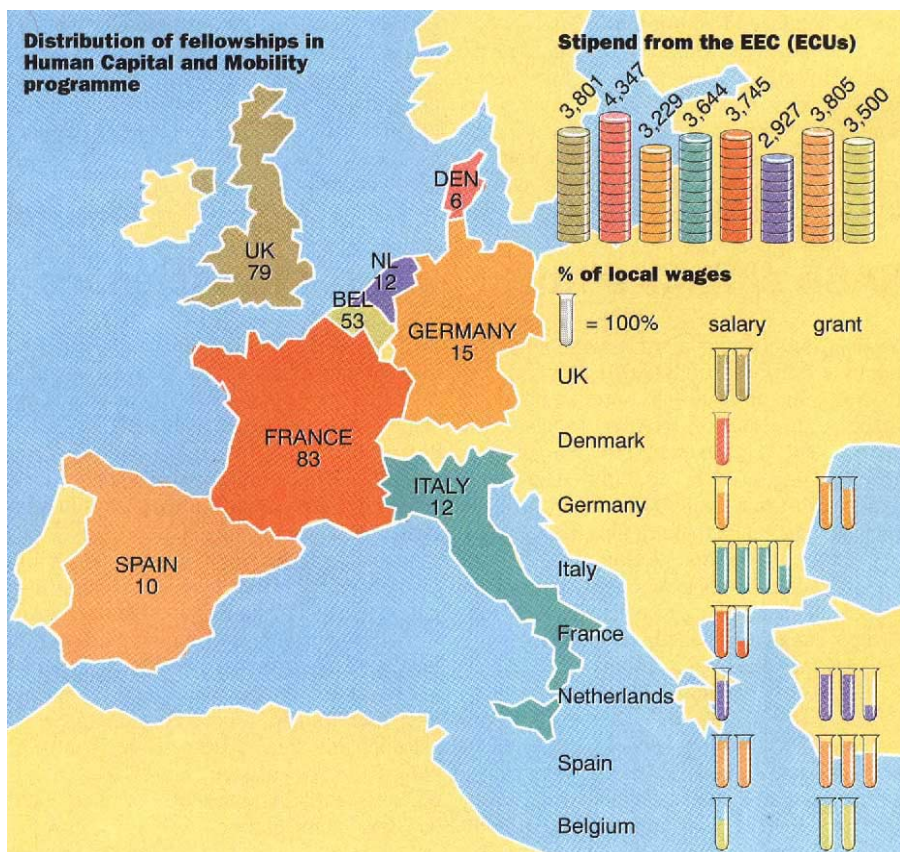
An initial decision by France to treat most fellowships as grants, for example, has been superseded by a recent agreement between the government and universities to consider them as short-term contracts subject to normal deductions. The EC is happy with the arrangement, which leaves the researcher with a take-home salary close to the local norm. But a special French law designed to cover unemployment costs following termination of such contracts means that any research institute or university may make an additional deduction of up to 10 per cent.

The changes have forced British geophysicist Jeremy Henderson to forsake a chance to work at the Institut de Physique du Globe de Paris. On the verge of leaving for France, he found out that he would be subject to deductions of 60 per cent — with an additional 20 per cent tax deduction to come. "My family couldn't live in Paris on a reduced salary when costs of accommodation are so much higher than here", he says.

Germany, Belgium and the Netherlands have as yet not decided whether the money should be given as salary or bursary. Full deductions mean a salary 20–30 per cent lower than local colleagues; no deductions make possible one that is double the local salary. Frans Martens of the Netherlands says that salaried contracts are preferable because they "ensure a good status" for the researcher but that take-home pay should match local conditions. In Belgium, the problem is complicated by the different regulations in the federal regions of Flanders and Wallonia.

German institutes have tried to find individual temporary solutions, leading to cases in which fellows could earn as little as a laboratory assistant or as much as a full professor. Some universities have washed their hands of the matter, giving fellows the full lump sum and leaving them to take on the complex German tax and social security system — in a foreign language.

In Italy, direct comparisons are difficult because few short-term fellowships are is-



sued nationally and the pay tends to be very low. But the administration of the EC grants is relatively straightforward — a 20 per cent tax charge to the national research council and a 10 per cent charge to the university — and researchers do well financially, earning as much as four times the fellowship stipend or twice that of an equivalent permanent research worker.

British universities are also trying to make arrangements at a local level to avoid paying fellows the same salaries as department heads. For example, the University of Warwick holds back between a quarter and a half of the grant for travel and publishing (leaving the fellows very much better off than their bench mates), but the University of Cambridge was told that it could not ask fellows to return some of their cash to the university department for equipment or laboratory consumables. Salaries are exempt from tax in some universities.

Of host countries with the largest number of fellowships, only in Denmark is the programme working relatively smoothly. All fellows there are given their money on a contract basis, and their take-home pay does not deviate significantly from local levels.

But even if the question of payments is answered, a second problem is bound to arise. Researchers will naturally favour the countries where they will be best off, putting countries whose tax and social security laws force them to offer low salaries at a distinct disadvantage.

How could such an ambitious and well-funded scheme have foundered so disastrously on such obvious and predictable points? The European Commission has been criticized for refusing to become involved in solving the problems of the individuals it funds, but it is not the only culprit. The tortuous democratic processes of the European Communities must also share the blame, with proposals designed by the commission bounced to and fro among the European Parliament, the commission and the European Council of Ministers. The original proposal, for example, included provision of bench fees within the fellowship money, but it was eliminated to conform to the wishes of the parliament to avoid any direct support of research.

The commission erred in accepting salary figures from each member state without consulting those who must administer the grants, but its hands are tied to some extent by the decisions of the Council of Ministers. Although living costs and normal salary deductions were taken into account, the resulting scales are badly flawed.

The commission is working with member states to resolve the immediate problem of salaries, but a long-term solution to the disparity within and between countries will require a political decision. In the meantime, European scientists are trying not to let their frustrations override their enthusiasm for the principles behind the programme.

Alison Abbott

Congress searches for new role for energy laboratories

Washington. Two US lawmakers have proposed different solutions to the pressing problem of finding a civilian role for the research laboratories operated by the Department of Energy (DOE). One would encourage all DOE laboratories, not just those conducting weapons research, to cooperate with industry in fields ranging from the environment to health, advanced manufacturing, space and high-performance computing. The other would ask DOE to narrow the mission of individual laboratories and, perhaps, even to close some of them.

The first bill, introduced on 2 March by the chairman of the Senate Energy and Natural Resources committee, would require the laboratories to spend at least 10 per cent of their research dollars on cooperative projects with industry, in line with recommendations from both the Bush and Clinton administrations. The DOE spends about \$6.6 billion a year on research and development at more than 20 laboratories scattered around the country. The legislation, introduced by Senator J. Bennett Johnston (Democrat, Louisiana), would also simplify the paperwork and other requirements for making deals with private companies.

Not surprisingly, the directors of DOE's primary nuclear weapons research laboratories — Lawrence Livermore in California and the Los Alamos and Sandia laboratories in New Mexico — support any attempt to diversify their business now that the Cold War has ended. In a November letter to just-elected President Clinton, they offered their services in supercomputing, environmental monitoring and other fields where "science and technology can help make a difference" to the US economy.

Pete Lyons, the deputy director for energy and environmental research at Los Alamos, says that his centre would like eventually to spend as much as 20 per cent of its budget on partnerships with industry, a figure that Clinton mentioned during his campaign as a ceiling for such efforts. More than that, says Lyons, would be a mistake: "You can't sustain a laboratory on technology transfer."

In fact, critics of this approach claim that turning the energy laboratories into high-technology 'job shops' without specific responsibilities is a recipe for disaster. Instead, they say, the laboratories should have a few clearly defined 'core missions' against which their performance can be measured.

US Representative George Brown (Democrat, California), the chairman of the House Science, Space and Technology committee, has suggested that weapons research might be consolidated at one or two laboratories, with the others being converted pri-

marily to civilian uses such as improving the environment. Brown was expected this week to introduce a bill that would require DOE to report on how it might accomplish this cost-cutting and consolidation.

Brown may also ask for the creation of a laboratory closure commission similar to that established to decide which US military bases should be closed. But the politics of closing any government facility — particularly if it involves cutting high-technology jobs — may prevent this. Even realigning personnel is politically difficult, says Edward Frieman of the Scripps Institution of Oceanography, who chaired a task force last year that advised DOE on the future of its laboratories. Erich Bloch, former director of the National Science Foundation and a senior fellow at the nonprofit Council on Competitiveness, which conducted a similar study last year, says that the three weapons laboratories are "unkillable".

Not that Brown, or anyone else, has pinpointed which laboratories, if any, should be closed. But with some 700 federally run laboratories and a diminishing need for defence-related research, the government has a problem of oversupply. Meanwhile, agencies such as the Department of Commerce intend to create new centres to feed the Clinton administration's desire to transfer technology from government to the private sector. Next week, Energy Secretary Hazel O'Leary will testify before Johnston's committee about her vision for the laboratories.

Tony Reichhardt

India moves ahead on rocket contract

New Delhi. India has selected four private and two government companies to start making engine components using the cryogenic rocket technology that it is purchasing from Russia over the objections of the United States.

The \$80-million contract with Glavkosmos will enable India to build engines to power the upper stage of a rocket to launch 2.5-tonne satellites into a geostationary orbit. The United States has imposed a two-year ban on exports of space technology to the Indian space agency because it considers the contract to be a breach of an international agreement to prevent the spread of nuclear technology (see *Nature* 357, 99; 1992), but the Russian president, Boris Yeltsin, assured Indian officials last month during a visit that his country would abide by the agreement signed by the former Soviet Union. The first launch is planned for 1995.

K.S. Jayaraman

Kafatos to be EMBL director; promises greater opportunity

Munich. The new head of the European Molecular Biology Laboratory (EMBL) says that he will increase opportunities for women and for scientists from developing countries while increasing cooperative ventures with national research programmes.

Fotis Kafatos, a 53-year-old developmental biologist from Greece, appears to be a popular choice as the laboratory's third director-general. He is the founding director of the 11-year-old Institute of Molecular Biology and Biotechnology in Heraklion, Crete, and is also a full professor at Harvard University.



Fotis Kafatos

Appointed last week by the EMBL Council, Kafatos takes over on a part-time basis next month, assisted temporarily by John Tooze, now executive secretary of the European Molecular Biology Organization. Kafatos becomes full-time in February 1994, when he also will appoint a director of administration to allow more time for his own scientific work. His laboratory, including some of his team from Heraklion, will continue working on the developmental biology of the mosquito and the fruitfly. "The perspective of an active scientist is very important in directing an institute of this nature", he says.

Kafatos succeeds Lennart Philipson, whose resignation last July exposed deep

rifts between the laboratory and its council of 14 member states over the right of a single country to veto EMBL decisions (see *Nature* 358, 267; 1992). The resulting search has been the subject of much unhappy rumour and speculation.

Italy, for example, broke the convention of confidentiality during negotiations by threatening to leave EMBL or to sue the laboratory in international court if Kafatos was allowed to maintain links with his previous institutions. The threats were widely perceived as an attempt to promote the Italian candidate.

But Kafatos has made clear his commitment to the laboratory by resigning his position at Heraklion and by declaring that he will resign from Harvard at the end of his six-month sabbatical. He wants to reestablish a good relationship with member states, calling for a spirit of cooperation between the laboratory and the council to avoid "sabotaging EMBL and the cause of European unification in science".

Kafatos says that his goals can be summarized in three words: excellence, inclusiveness and cooperation. Scientific excellence rather than simple growth is the key to success, he says, in a reversal of a policy of expansion that brought Philipson into conflict with member states hit hard by recession. His commitment to inclusiveness — an acceptance of talent without regard to gender or nationality — should please those who have long felt that women are treated unfairly at EMBL. "Until a couple of decades ago, male scientists were usually oblivious to the talents of even the most remarkable female scientists", he says. Similarly, Kafatos sees

scientists from less wealthy European countries, such as his own homeland, as an untapped source of talent.

His wish for closer cooperation between EMBL and member states in scientific ventures has a pragmatic side as well — improved access to outside funds. "If British biology is being rejuvenated by the Wellcome Trust, shouldn't equivalent sources be accessible to the premier international centre of molecular biology in a unifying Europe?" he asks. **Alison Abbott**

EMBL selects Cambridge as site for new database

Munich. The council of the European Molecular Biology Laboratory (EMBL) last week chose Cambridge, England, as the site of a new European Bioinformatics Institute (EBI). EBI will extend the services of EMBL's current data library, primarily a nucleotide and protein sequence database, which has grown too large for EMBL's central laboratory facilities in Heidelberg, Germany.

The decision is a disappointment to the rival bidders, Heidelberg and Uppsala, Sweden (see *Nature* 361, 383; 1993). EBI planning officer Howard Bilofsky said that the losing bids were "highly credible" but that the "scientific, intellectual and technical environment offered by Cambridge was ideal". Particularly important for the venture are the high-speed data links connecting Cambridge with the rest of the world.

About 70 scientists and technicians will in the next two years move into a custom-designed building on the genome campus at Hinxton Park, nine miles south of Cambridge, already home to the new genome research institute, the Sanger Centre. Some will be drawn from the existing staff of 40 at the data library in Heidelberg, while others will be recruited.

EBI will not raise the cost to member states of participating in EMBL. The US\$12 million needed for the facility will be provided by the British Medical Research Council and the Wellcome Trust. EMBL also plans to hold steady its DM5-million (US\$3 million) contribution to operating the library, which will cost DM12 million annually. EMBL hopes that the European Communities will increase their support to DM5 million and that the remaining DM2 million can be obtained through research grants and contracts with industrial users and others such as the European Patent Office.

EMBL says that services will not be interrupted during the move and that the data library — which each week handles more than a hundred sequence submissions and several thousand requests for data — will not be wound down until it is replaced.

Alison Abbott

Ruberti modifies fourth Framework

Munich. The research minister for the European Communities (EC), Antonio Ruberti, has put his own stamp on proposals for European-wide research projects from 1994 to 1998 while endorsing the recommendations of his predecessor, Filippo Pandolfi, for the fourth Framework programme (see *Nature* 359, 471; 1992).

Ruberti has proposed greater integration with national programmes and with the work of bodies such as the European Molecular Biology Organization, the European Space Agency, the European Science Foundation and the European particle physics laboratory (CERN). He has called for regular meetings of the European research ministers and the heads of national and European research laboratories, a process that the laboratory directors have already begun (see

Nature 361, 576; 1993). He would also like to add projects on transportation and socioeconomic research to the programme.

Ruberti's changes are in line with the recommendations last December of the European Council, which also complained about the cost of Pandolfi's plans. It wants the proposed budget reduced from ECU14.7 billion (US\$20 billion) to between ECU11 billion and ECU8 billion. The third Framework Programme cost ECU6.6 billion.

Ruberti admits that the procedures for making decisions are "unwieldy" and that the system must be more flexible. The fourth Framework should also include a programme of technological assessment, he says, to strengthen its industrial base and to match similar programmes in the United States and Japan. **A.A.**

Canada decides, at last, to increase spending on AIDS

Quebec. Canada will increase AIDS spending in the next five years by 13 per cent in a decision that came just days before the end of funding for the current five-year plan for a national AIDS strategy.

The increase from C\$37 million (US\$30 million) to C\$42 million announced last week falls short of the C\$55 million requested by AIDS groups, but it relieves some of the anxiety caused by the government's failure to announce its plans earlier. Health Minister Benoit Bouchard had previously told the AIDS groups without explanation that their demand was unrealistic.

Despite the increased funding, there is sharp criticism of the government's approach to the crisis, which has seen the number of AIDS cases double in the past three years to about 12,000.

"Canada's government spends per HIV person about 40 per cent of what the US and Australia each spend", says epidemiologist Martin Schechter of the University of British Columbia, a prominent AIDS researcher and member of a Royal Society of Canada panel that in 1988 carried out a study requested by the federal health department. "The Royal Society report recommended spending \$80–\$100 million a year. The government's figure of \$42.2 million doesn't appear to be based on any kind of rational approach to the problem."

Money for AIDS research comes through a dedicated funding initiative authorized by the cabinet. It is distributed through various arms of the health department and the Medi-

cal Research Council of Canada (MRC). MRC devotes part of its own budget to AIDS research and training and has spent C\$13 million over the past five years.

The new five-year plan includes C\$17.8 million a year for "research and epidemiological monitoring", C\$6.2 million for education and prevention, C\$9.8 million for community development and support to nongovernmental organizations, C\$5.4 million for care, treatment and support and C\$1.5 million for coordination and collaboration.

The leader of Canada's clinical trials programme, Julio Montaner of St Paul's Hospital in Vancouver, is concerned about the country's ability to compete for the latest experimental drugs if there are insufficient funds for new clinical trials. Montaner has pointed out that similar groups in the United States, Australia and Europe "have resources several times greater than ours" and thus are more attractive to companies that want to test their products.

The health department said the second phase of its AIDS strategy will place greater emphasis on five areas that emerged last summer after consultations with activists: partnerships among federal, provincial and international agencies and private groups; recognizing HIV disease as a chronic and progressive condition; promoting health for people with AIDS and HIV; creating supportive social environments and promoting and sustaining healthy behaviour.

David Spurgeon

Italy resolves fight on space funding

Munich. A two-year fight over how much Italy's space agency ASI should give to basic science was resolved last week, freeing millions of dollars held hostage by the dispute.

The controversy hinges on an interpretation of a requirement that 15 per cent of ASI's total budget, which this year is IL800 billion (US\$500 million), should go towards fundamental research. ASI's president Luciano Guerriero believes that ASI's contribution to the European Space Agency should be counted in that percentage, which this year amounts to IL120 billion, but Remo Ruffini, chairman of ASI's grant reviewing committee, wants the share to be assessed from the whole budget and has emphasized his point by blocking all grant approvals (see *Nature* 356, 647; 1992).

A committee of scientists that was asked last month to resolve the issue (see *Nature* 361, 675; 1993) has sided with Guerriero, agreeing that the 15 per cent should include the portion of ASI's IL602 billion contribu-

tion to ESA that goes to basic science. This year that amount is IL112 billion, according to ESA president Jean-Marie Luton, leaving only IL8 billion for national projects.

The committee believes that it is unwise to give money to ESA but not to spend enough to reap the benefits of its contribution — that is, scientific payloads on ESA missions. Its recommendation for an additional IL40 billion for this purpose has been accepted by ASI and the ministry of research.

Franco Paccini, a member of the grant reviewing committee, calls the compromise "sensible and reasonable", but not everyone is satisfied. Ruffini believes that science has been shortchanged, and Riccardo Giacconi, head of the European Southern Observatory, has complained in a letter to the research minister that the agreement effectively cedes control over Italy's national research budget to the member states of ESA.

Alison Abbott

Amersham to pay £50 million for US Biochemical

London. Amersham International plc, the British health sciences group that was privatized in the early 1980s, is to buy United States Biochemical Corporation (USB) in Cleveland, Ohio, in an attempt to become a leader in the field of DNA sequencing products. The purchase price is just short of £50 million (US\$69 million).

The merger of the two companies will bring together Amersham's manufacturing and marketing experience in radioisotopes and molecular labelling, particularly in Europe and the Far East, and USB's strong research base in the enzymology of DNA sequencing and its knowledge and experience of the US market. Amersham's life science division had a turnover last year of £92.7 million, a third of the company's overall sales, while USB had sales last year of \$25 million.

USB, founded and still led by Thomas Mann, was already a major supplier of biochemicals and enzymes to the US life science industry when in the early 1980s it made a major commitment to molecular biology. In particular, two members of its scientific advisory board — Charles Richardson and Stanley Tabor of Harvard University — developed the enzyme Sequenase, which occupies a dominant position in the sequencing reagent market. USB holds the exclusive licence to the patent on the enzyme, which is owned by the university.

Amersham was originally part of the UK Atomic Energy Authority, supplying radioisotopes for research, and was one of the first companies privatized by Margaret Thatcher, the former prime minister. In recent years it has expanded into molecular biology, and it expects the merger with USB to give it a major slice of the sequencing market, now estimated to be worth \$100 million a year.

The British company is particularly interested in tapping into the growing demand for automated sequencing. Company officials point out that political and scientific interest in large-scale sequencing initiatives such as the Human Genome Project have led to growing pressure to reduce costs and to increase the productivity of sequencing procedures.

Its strategy assumes that no radical innovations in the technology of sequencing are likely for several years and that the best approach at present is to increase the effectiveness of the reagents used with current sequencing techniques. The acquisition of USB's portfolio of patents and patent applications for enzymatic nucleic acid sequencing forms a core element of this strategy.

David Dickson

The cost of surrogacy

SIR — Whether I am “a controversial figure”, as Declan Butler says (*Nature* 361, 102; 1993), I don’t know, but I did not “[create] an agency for surrogate mothers”.

As I have stated in several scientific papers and also in my book *Mères Porteuses, Oui ou Non?* (Frison-Roche, Paris, 1990), I set up a not-for-profit organization called Alma Mater whose aim was to manage the financial costs raised by surrogacy for those concerned. So far as I am aware, this organization is unique, although it resembles those involved in the adoption of children and indeed was recommended later in a leading article in *Nature* (320, 95; 1986). Should we be blamed for having pioneered an approach advocated by *Nature*?

As far as the CNRS group is concerned, our sperm bank, thanks to which this work was made possible, was indeed a “private” bank, but once again was not for profit. Contrary to allegations made in *L’Express*, we followed the correct procedures insofar as the law allowed us to do so. It is true that parents’ consent was not *totally* informed, but this was to ensure the utmost protection to those concerned. As Butler points out, the present state of the law is unsatisfactory for researchers, who should be able to have access to medical data without breaking the law, and also for parents, who must be protected. “Disobedience may be a duty”, said General Charles de Gaulle when he flew to London after the fall of France in 1939. That may also be the case for us.

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Failing forecasts

SIR — According to your leading article (*Nature* 361, 191; 1993), economists cannot be blamed for the forecasting failures that result from their models. After all, they have to make guesses about the behaviour of people and government.

In fact, their guesses are not random. They are based on assumptions that are psychologically implausible. Most prominent among these is the doctrine of Rational Expectations¹. According to this view, the expectations on which people base their behaviour are formed by making optimal use of available information. When dealing with noise-free systems, they have perfect foresight; when dealing with noisy ones, their forecasts cannot be bettered. Econometric

models are based on this assumption². For the deputy governor designate of the Bank of England, its adoption represents the most significant recent development in macroeconomics³. What is so ironic is that the models that produce such clearly suboptimal forecasts are based on the assumption that people make optimal forecasts.

The assumption that people are substantively rational was not made by Keynes but by economists who succeeded him. For many years, experimental psychologists have demonstrated that it is unreasonable⁴. Reconsideration of Keynes’s ideas may have been forced on us by the need find ways to deal with the recession. However, it may also signal a growing impatience among economists with unrealistic assumptions about the rationality of human behaviour⁵.

However good the assumptions, inadequate datasets will always lead to forecasts that are prone to error. The reason that economic forecasters appear to promise too much is that their predictions are presented in the media as deterministic. No quantitative estimates of the levels of uncertainty associated with them are provided. In contrast, weather forecasts are increasingly presented probabilistically by well-calibrated meteorologists⁶. It rains on 70 per cent of the occasions on which forecasters say that there is a 70 per cent chance of rain. Given this, the public is not inclined to ridicule them on the 30 per cent of occasions that this forecast is given but it does not rain. They have not promised too much. Perhaps economic forecasters could learn from their example.

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Global warming

SIR — It is accepted that the burning of tropical rain forests can be a significant contributor to an increase in global atmospheric carbon dioxide, with consequent implications for potential global warming. Obviously the most immediate and direct way to reduce net forest-related carbon dioxide emissions is to work to restrict global forest burning, particularly in rain forests. However,

planting new trees can also improve the net balance of carbon dioxide.

Carbon sequestration potential varies by species, but average carbon storage for mature woodland in Britain is about 60 tonnes per hectare¹. A recent but conservative estimated world damage value per tonne of carbon is \$10 (ref. 2). This suggests an eventual economic value of \$600 per hectare of mature woodland in prevented global warming damage.

This year’s European Communities (EC) agreement requiring farmers to set aside 15 per cent of their land in order to be eligible for EC support prices offers an economically favourable opportunity for carbon sequestration. But the agreement unfortunately specifies that the land set aside must be rotated back into agricultural production every subsequent year; this makes afforestation impossible as a set-aside option. Even worse, British farmers currently participating in the earlier 1988 national set-aside scheme, where afforestation was permitted and encouraged under the Ministry of Agriculture’s “Woodland Option”, were informed in September that this land could not be counted towards their new 15 per cent set-aside requirement. The current incentive is to withdraw from the 1988 scheme and to tear out previous tree plantings in order to comply with the new regime’s rotational requirement.

European set-aside, as it is now conceived, contributes little to nature conservation or environmental protection. If we look on set-aside as an opportunity to do more than just reduce agricultural production, we may wish to allow and encourage afforestation. More radically, we could in some cases allow succession to take its course on set-aside land. Such a policy has produced extensive areas of secondary woodland in the United States, but in Britain we seem to have largely forgotten that tree planting is often unnecessary to produce woodland succession³. Imaginative set-aside could, as well as reducing agricultural surpluses, offer us a nature conservation and carbon sequestration bonus.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Why microtubules grow and shrink

A new model by two physicists of the assembly of microtubules from the monomer tubulin throws an interesting light on the regulation of this important and ubiquitous process.

THIS journal's view that molecular biologists owe it to themselves and to their subject to make molecular biology more quantitative is occasionally vindicated in the most unexpected ways. One such occasion is the appearance, in *Physical Review Letters* for 8 March, of an intriguing discussion of the dynamics of the growth and, conversely, the shrinkage of microtubules, the structural elements that determine the shapes of cells and which are also involved in the rearrangement of chromosomes at mitosis, for example. Although the argument is purely theoretical, it has the merit of showing up one of the most characteristic features of this system — the delicate balance there seems to be between the growth of microtubules and their shrinkage.

The assembly of a microtubule is now, in its bare bones, understood. The raw material is the protein tubulin, which can be made to polymerize to form arbitrary lengths of a microtubule. But the process is not straightforward. Nearly a decade ago, Tim Mitchison and Marc Kirschner, then both at the University of California, San Francisco, showed that, in the same *in vitro* preparations, some microtubules can be growing while others are shrinking (*Nature* **312**, 232–237 & 237–242; 1984).

Mitchison and Kirschner coined the term "dynamical instability" to describe this odd behaviour and offered as an explanation that monomers near the growing tip of a microtubule are loaded with GTP, the energy of which is required for polymerization. The result of the addition of monomers is that the GTP is converted to the diphosphate GDT. The result is that a growing polymer will tend to continue to grow, but that once it has started to shrink, it will also continue in that state. A practical consequence, which may be significant within the cell, is that some polymers will continue to grow even though most others are shrinking. More generally, dynamic instability tends to broaden the length distribution of the polymers with the passage of time.

What Marileen Dogterom and Stanislas Leibler from Princeton University and the Theoretical Physics Unit at Saclay, at Gif-sur-Yvette near Paris, have now done is to clothe the Mitchison and Kirschner in mathematical notation (*Phys. Rev. Lett.* **70**, 1347; 1993), but even that provides a degree of insight into the dynamics of the growth of microtubules that has a value of its own, the inevitable crudeness of the assumptions notwithstanding.

This is how the argument goes. For the

centrosomes to which microtubules are anchored during mitosis, and which are spherical structures built from protein monomers, Dogterom and Leibler suppose that one end of the microtubule is anchored to a flat surface. To simplify the geometry, they suppose that all microtubules, whatever their length, are rigid and that they grow perpendicular to the anchor-plate.

The essence of the model must be a mechanism for switching between the states of growth and of shrinkage, which is supposed to be a random process, with frequencies f^+ (for switching from shrinkage to growth) and f^- (from growth to shrinkage). Similarly, the speed with which microtubules grow and shrink are represented by v_+ and v_- respectively. The rate of growth will be influenced by the concentration of tubulin near the tip of a growing polymer, but the second is supposed to be a constant. The physical chemistry of tubulin polymerization shows that v_- may be an order of magnitude greater than the assembly speed and of the order of 20 μm a second.

As it happens, the problem can even be dealt with analytically, at least if it is supposed that many microtubules grow out of the same flat anchor-plate and if, on the average, they are further apart than the characteristic diffusion length of tubulin (so that they will not compete for monomer). Then, conceptually, it is possible to define the probability density of growing and shrinking microtubule tips as a function of distance from the anchor-plate. (For example, the probability density of growing tips, p^+ , at a distance z from the plate will describe the number of growing tips in an infinitesimal interval z ; thus both p^+ and p^- are functions of z and of the time, t .)

The upshot (assuming no competition for monomers between neighbouring polymers) is that the parameters of the problem define a sharp transition between a state in which there is unbounded growth (the average length of polymers increases with time, linearly as it happens) and one in which there is a steady state — some microtubules continue to grow, but others shrink at such a rate that the average length remains constant. The transition is defined by the condition that $v_- f^- = v_+ f^+$. The statistical properties of the assembly of polymer chains can then be calculated and, in an elaboration of the model, the authors even go on to allow for competition between the growing polymers through their competition for monomer, the distribution of which is regulated by the speed with which it diffuses

through the space between the growing or shrinking polymer chains.

Nobody claims that these results are more than a framework within which the general character of microtubule assembly can be discussed with a little more precision than has previously been the case. But it is plain that there are several ways in which the rate of microtubule assembly can be critically affected by small changes in the parameters of the problem, which themselves could well be determined by variations of the condition of the cell at different stages of the cell cycle.

For example, the rate at which shrinking polymers start to grow again (f^+) may well depend on the concentration of monomer, or at least on the concentration of monomer loaded with GTP, which may well itself be regulated by the activity of some phosphorylase. Or the speed of assembling polymers may be more sensitively related to the concentration of monomers than the linear relationship assumed. The plain import of the model seems to be that for microtubules to grow at all requires that exacting conditions should be satisfied. Then, small variations of any one of the parameters will send the system already in dynamic instability into some other condition altogether.

A further suggestive point arises from the authors' attempts to allow for the finite speed of monomer diffusion on the growth of a bundle of parallel microtubules. The point is that the supply of monomer near the still flat anchor-plate will have been depleted by the passage of those polymers that have already grown through the region, so that shorter polymers will have to grow with the benefit of a reduced supply of the components required for their assembly. In suitable circumstances, the result is that the population of microtubules growing from a fixed (and still planar) anchor-plate is split into two — those whose tips are already in the region where monomer is plentiful, and those which are left behind in the depleted region, and which are destined for repeated brief cycles of assembly and disassembly.

Nobody suggests that any of this implies that the dynamic instability of microtubules has now been made a matter of simple (or even complicated) arithmetic. That is not the function of models, at least in complicated circumstances such as those in which real-life microtubules are assembled. Rather it is that models have a heuristic value, directing attention in this case to those quantities that are likely critically to affect the behaviour of a system. That this model does excellently.

John Maddox

Voyage of an ancient *mariner*

Margaret G. Kidwell

*It is an ancient Mariner
And he stoppeth one of three.*

QUESTIONS about the origin and evolutionary age of mobile genetic elements have been around since Barbara McClintock first described 'controlling elements' in maize almost half a century ago. Now, with the help of the polymerase chain reaction (PCR), answers are beginning to emerge. The report by Hugh Robertson on page 241 of this issue¹ strongly suggests that the mobile *Drosophila* element, *mariner*, is of ancient origin. Robertson also describes a broad, but patchy, distribution of this element family, together with some unexpected relationships among *mariner* elements from the same and different species of host insect.

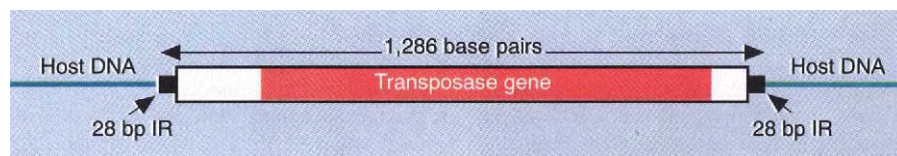
The *mariner* element was first identified in *Drosophila mauritiana*². This species is restricted to the island of Mauritius and is a close relative of that genetic work-horse, the cosmopolitan species *Drosophila melanogaster*. At a little over one kilobase in length (see figure), members of the *mariner* family are relatively small³. They belong to a major class of transposable elements which have short inverted terminal repeats and differ from members of the other major class, the retrovirus-like elements, in using a DNA, rather than an RNA intermediate in transposition.

At the time of their discovery, only eight years ago, there seemed to be little reason to think that *mariner* elements would be any more widespread than the isolated species that carry them. More recent studies have indicated that these elements are broadly distributed in species of the melanogaster subgroup of *Drosophila*, although they are absent from *D. melanogaster* itself⁴. However, in more distantly related drosophilids, strongly hybridizing elements were detected only in a single species *Zaprionus tuberculatus*, which is estimated to have diverged from *D. mauritiana* at least 50 million years ago⁴.

Now Robertson¹ has extended the search far beyond the Diptera. A previous serendipitous discovery of *mariner*-like sequences in the cecropid moth *Hyalophora cecropia*⁵ allowed him to design degenerate PCR primers to the only two conserved regions of contiguous amino-acid similarity between the *mariner* elements of *D. mauritiana* and *H. cecropia*. He reports the presence of these elements in ten other species, representing six additional orders, including insects as diverse as bees, mosquitoes, silverfish, cat fleas and earwigs.

Four principal subfamilies of *mariner*

elements have been identified so far, with the promise of more to follow. One new significant finding is the clear indication that *mariner* is very old; some of the subfamilies were apparently differentiated before the divergence of their arthropod host lineages 200–300 million years ago. In like fashion, similarities between predicted proteins of the *D. mauritiana mariner* element and the *Tcl*



Structure of an active *D. mauritiana mariner* element inserted into host DNA. A single open reading frame constitutes the transposase gene. IR indicates inverted terminal repeats.

element from the nematode *Caenorhabditis elegans* have also been recently verified⁶.

The presence of several subfamilies of *mariner* elements in several individual insect species is puzzling, as is the observation that some distantly related insects carry closely related *mariners*. Early divergence of the subfamilies with subsequent loss in some descendant species, together with occasional horizontal transfer across species boundaries, may provide the most likely explanation of the observed patterns. But other hypotheses to explain these results cannot yet be ruled out.

Among transposable elements, the *mariner* family seems not to be alone in its antiquity and broad, somewhat unorthodox, current distribution. For example, the *Ty1-copia* and *Ty3-gypsy* retrovirus-like transposable elements also appear to be very old and to be ubiquitous in plant genomes^{7,8}. Like *mariner*, some incongruities between the distribution patterns of these retrotransposons and their host phylogenies have implicated occasional horizontal transfer, sometimes between species in different kingdoms. The essential nature of 'jumping genes' may make them more prone than non-mobile genes to hop across species boundaries. This capability may also promote their long-term evolutionary survival.

These observations are not only of basic biological interest, but also have implications for potential applications. There is considerable interest in the development of generalized DNA vectors for the transformation of insects. However, the *Drosophila* P element that was used in pioneering experiments to develop this technology⁹ unfortunately has a restricted host range; conversely,

although the host range of some retrovirus-like elements is broad, other properties make their use for this purpose questionable.

Prospects for the application and development of *mariner* in genetic engineering are altogether more promising. In addition to its structural similarity to the P element, active *mariner* elements from *D. mauritiana* can integrate, in a stable fashion, into the germ line of another species from which they are naturally absent¹⁰. So the newly revealed broad host range of *mariner*-like sequences augers well for their possible use

in tagging and cloning and for their development as effective transformation vectors over a wide range of arthropods.

Although some broad patterns in these new results seem to be emerging, caution in their interpretation is called for pending a better understanding of the evolution of multigene families. Transposable elements may evolve at faster rates than single copy genes, or may evolve differently in other ways. But can differential rates, or modes, of evolution explain the existence of unusually diverse elements in the same genome? How often does horizontal transfer of DNA sequences occur? What is the nature of the vectors involved? Is the host species merely a passive observer of the sometimes frenetic activity of parasitic DNA? Alternatively, do host and element genomes interact together for their mutual benefit? As usual, as old questions are settled a fresh and larger crop of them arises:

*But tell me, tell me, speak again,
Thy soft response renewing —
What makes that ship drive on so fast?
What is the ocean doing?*

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One cloud and its silver lining

J. D. Lowenthal

DISTANT, luminous quasistellar objects shine through multitudes of otherwise invisible intergalactic clouds of hydrogen that carve a forest of Lyman- α absorption lines out of the background sources' spectra. Unfortunately, because the background sources are typically point-like, we usually get no information on the size or form of the foreground clouds: are they flat or round, the size of galaxies or of the Solar System, neutral or ionized? On page 224 of this issue¹, however, Hippelein and Meisenheimer present the results of a clever observation in which they imaged in absorption a putative Lyman- α forest cloud in front of a distant radio galaxy; because radio galaxies are spatially extended, this experiment yields a direct measurement of the cloud's physical shape and size, which corresponds roughly to the dimensions of large local galaxies.

The issue bears strongly on various pictures of galaxy formation and cosmology: because of the finite speed of light, we see the Lyman- α clouds as they were when the Universe was very young, so that knowing their basic physical parameters will clarify their relation to normal galaxies. It will also provide insight into such important issues as the strength and source of the intergalactic ionizing radiation field.

The size of Lyman- α clouds is crucial for understanding their ionization states, abundance of elements heavier than helium (metals) and gas densities, but cannot be obtained by analysis of the spectra of the quasistellar objects (QSOs). The multiple images of rare, gravitationally lensed QSOs offer one way forward. Each image corresponds to a distinct path followed by the QSO's radiation. A small cloud may appear on a single image, but a large one may intercept several paths so that its size may be estimated. The most carefully studied² gravitationally lensed QSO case to date, UM673 A and B, yields typical cloud sizes in the range 10–200 kiloparsecs, depending on the interpretation of a few particular absorption lines and on cosmological parameters. This agrees with earlier similar studies³ and is reminiscent of normal galactic dimensions today. But a contrasting suggestion, based on a possible correlation between Doppler spectral line widths and column densities, and on the observed narrowness of some absorption lines⁴, is that the clouds are cold and minuscule (less than a parsec) or sheet-like^{5,6}.

Hippelein and Meisenheimer's observations make use of two important characteristics of the Lyman- α emission

line in the spectrum of the luminous, high-redshift ($z = 3.4$, corresponding to a lookback time of at least 80 per cent of the age of the Universe) radio galaxy 4C41.17 (ref. 7). First, the radiation comes from a spatially extended region, subtending some 10×13 arcseconds on the sky, or about 100 kiloparsecs at the radio galaxy's distance from us; and second, the emission line is spectrally very broad. Together, these facts cause the extended emission line region to appear as a light screen, a backdrop resembling a continuum source, against which any intervening neutral hydrogen cloud within a reasonable distance of the radio galaxy would appear as a dark Lyman- α absorption feature; if the intervening cloud were spatially extended, so would the absorption feature be. This is exactly what Hippelein and Meisenheimer report, in agreement with a previous, detailed study of the 4C41.17 system⁷.

Using a Fabry-Perot interferometer (a tuneable narrow-band filter) and an imaging charge-coupled device (CCD), Hippelein and Meisenheimer took images of the Lyman- α emission line, stepping the Fabry-Perot through consecutive wavelengths, mostly blue-shifted from the emission line (to see intervening absorption lines). At the wavelength corresponding to Lyman- α at a blueshift of about 360 km s^{-1} with respect to the emission-line maximum, they detect an oblong dark patch visible against the bright background. They propose that it is due to an intervening hydrogen cloud of dimensions 10×14 kiloparsecs, which could be 5 megaparsecs from the background source.

Assuming some nominal Lyman- α cloud parameters, Hippelein and Meisenheimer derive a column density of neutral hydrogen around 10^{15} cm^{-2} , yielding for their measured cloud size a total mass of hydrogen of 3×10^7 solar masses. This would imply that the Lyman- α forest clouds at high redshift are the same size as modern-day galaxies, but much less dense, and are perhaps held together by their own gravity⁸. They could be elongated or possibly sheet-like, and have 10^4 – 10^5 times as much ionized as neutral hydrogen. This picture is in good agreement with the implications of the earlier gravitationally lensed QSO studies⁴. The presence of any absorption lines due to metals would show that the cloud is not pristine and has been contaminated by a generation of star formation.

However, several caveats remain. In particular, the interpretation of the dark

patch is far from unambiguous. As Hippelein and Meisenheimer point out, it could be due to complexities in the background emission (indeed, the Lyman- α emission cloud surrounding the radio galaxy is already known to be quite complex kinematically⁷). And as the authors recognize, even if the cloud is real, it might be strongly influenced by radiation from the powerful radio galaxy. Or it might even be directly associated with the radio galaxy itself and so not typical of Lyman- α forest clouds at all. This last possibility is supported by previous long-slit spectroscopic images⁷ of the Lyman- α line, which give higher resolution than do Hippelein and Meisenheimer's narrow-band images. As one would expect from the images, a dark absorption (or at least a lack-of-emission) feature is visible a few angstroms blueward of the emission line peak. The absorption line appears to have a velocity gradient of 120 km s^{-1} , typical of galaxy rotation effects but larger than typical Lyman- α cloud velocity widths. Chambers *et al.*⁷ refer to this feature as a disk, and it is presumably the same object or component that Hippelein and Meisenheimer imaged. The inferred mass, if the velocity shear represents a circular orbit driven by gravity, is around 3×10^{11} solar masses (or a bit less, depending on the inclination of the disk to the observer); this is clearly different from the mass given by Hippelein and Meisenheimer.

There are several other high-redshift, luminous radio galaxies with extended regions of Lyman- α emission: if these typically show dark patches similar to that in the spectrum of 4C41.17, the natural conclusion would be that the absorbing material is linked directly to the parent galaxy, either in the form of a surrounding ring or shell or as a companion galaxy or cloud. If so we would stand, at least, to learn much about distant radio galaxies, which may in turn hold clues to normal galaxy formation. But if the absorbing cloud turns out to be representative of the Lyman- α forest, the implications for our understanding of the clouds' physical parameters will be far-reaching. □

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RÉSUMÉ

Tell tall

M. PETRIE and A. Williams went to work on an egg, or rather 519 of them, to test a prediction of life-history theory (*Proc. R. Soc. Lond. B* **251**, 127–131; 1993). The prediction is that if females gain in one way or another from mating with attractive males, they are likely to invest more in reproduction with such males. The eggs were those of peahens. They were laid by a total of 32 birds which had been split into groups of four, each of the groups being put into a pen with a single male. It turned out that significantly more eggs were produced for peacocks with larger masses of train feathers (mass was one of several possible measures of train elaboration). That females do indeed invest more in reproducing with more attractive males has been observed before, but not in a species such as peafowl in which males do not feed the female during courtship or help care for the offspring.

Anybody there?

THE search for extraterrestrial intelligence (SETI) may have more chance of success than those engaged on the project say (J. F. Kasting, D. P. Whitmire & R. T. Reynolds *Icarus* **101**, 108–128; 1993). For a planet to sustain life, it must be not so near the parent star that its water content is boiled and photolysed away; but nor should it be so far away that atmospheric carbon dioxide (necessary as a greenhouse gas) condenses to form clouds, shading the surface. With these twin conditions, the authors estimate the size of habitable zones around various types of star (fortunately, Venus and Mars fall outside the limits for the Sun). Hotter 'F' stars evolve too quickly for life to develop, but planets around cooler 'M' stars become tidally locked on their orbits, so that one hemisphere always looks out to cold outer space. K stars, however, seem to be even more favourable than the Sun-like G-stars, currently targeted by the SETI project, and should be included in the search, the authors argue.

In the drink

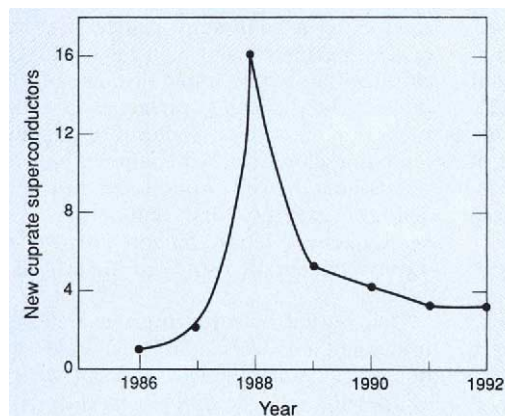
THE happiest experimental animals may be the strains of ethanol-sensitive mice that are indulged in their little weakness by G. S. Wand *et al.* (*J. biol. Chem.* **268**, 2595–2601; 1993). In cells *in vitro*, as well as in animals, ethanol intoxication represses the adenylylcyclase pathway with readily imaginable consequences on neurotransmission. GTP- and agonist-stimulated adenylylcyclase activity in membranes of the mouse brain is found to fall. Wand and colleagues now show that a superabundance of the electric soup increases the expression of a pair of inhibitory G proteins, but the one responsible for stimulation is unaffected. A sobering thought!

SUPERCONDUCTIVITY

Pick your poison

R. J. Cava

INVARIABLY, the first question colleagues ask if we haven't seen each other in a few months is "what's new in high- T_c ?" Like me, they remember the glory days when every week held the possibility that a new superconducting copper oxide would be discovered, and they would like to find out if there is anything new and exciting to hear. Although the level of worldwide effort in research into high-temperature superconductors is still reasonably high, the



Rate of discovery of new, structurally or chemically distinct cuprate superconductors.

pace of dramatic discovery has slowed significantly in recent years. We find ourselves in a period of more conventional scientific process, as the rapidly maturing field advances by evolutionary rather than revolutionary steps. The announcement of the new 94-kelvin superconductor $\text{HgBa}_2\text{CuO}_{4+\delta}$ by S. N. Putilin and co-workers in this issue (*Nature* **362**, 226–228; 1993) brings back some of the old flavour of expectation and potential that has been lacking in the superconducting-materials business of late.

Although commonly viewed as a revolution in solid-state physics, high-temperature superconductivity has been even more of a revolution in materials science. It has, after all, been driven from the very beginning by the discovery of new materials, not by either theoretical guidance or the understanding of the microscopic physics of the superconductivity. The new materials have followed a continuous path towards increasing chemical, synthetic and structural complexity in the past six years. It is no accident that the simplest materials were discovered earliest: the explosive increase in the superconducting onset temperature (T_c) that followed was a consequence of the explosive growth in knowledge in the materials-science community of the empirical chemical and

structural factors necessary for high-temperature superconductivity to occur.

Unless your speciality is finding new superconducting materials, the number and complexity of known copper-oxide-based superconductors has reached the point where it is difficult to keep up. It is no help that many published reports claiming to reveal 'new' superconducting copper oxides are either completely wrong, or represent only the partial substitution of one atom for another in a trivial way in a superconductor that is already well known. Including the new $\text{HgBa}_2\text{CuO}_{4+\delta}$ superconductor announced in this issue, by my count there are 35 chemically or structurally unique copper-oxide superconductors known to date. The pace of discovery of those superconductors is shown in the figure. In the remarkable peak year of 1988, 16 new cuprate superconductors were discovered, a majority of which were members of families of compounds based on bismuth-oxide and thallium-oxide intermediary layers. The pace since 1989 has slowed to about four per year: almost without exception these have been more complex, more difficult to synthesize, and have lower transition temperatures than those found throughout 1988.

If viewed from a jaded perspective, the addition of one more superconductor to the list would not seem to make much difference. $\text{HgBa}_2\text{CuO}_{4+\delta}$ has, however, several qualities that distinguish it from other cuprate superconductors discovered of late. Most importantly, it is apparently easy to synthesize and structurally simple, bucking the now well-established trend towards difficulty and complexity. These qualities will greatly facilitate experimentation with it for potential applications. Secondly, its transition temperature of 94 K is very high, especially for a superconductor based, as it is, on a single copper layer. As the authors point out, members of this family based on double or triple copper layers, when synthesized and correctly processed, should have even higher transition temperatures, perhaps in the vicinity of 125 K, which are accessible at present only in thallium-oxide-based materials. Current efforts in the development of bulk applications at liquid-nitrogen temperature for superconductors centre around $\text{YBa}_2\text{Cu}_3\text{O}_7$ and bismuth-oxide-based cuprates, with transition temperatures near 90 K; in spite of their very high transition temperatures,

the thallium-oxide-based superconductors face several problems which have slowed their development. Perhaps this, or another member of the mercury-oxide-based cuprate family, will have very high transition temperatures without those problems.

No doubt it has not escaped your notice that trading mercury for thallium is probably just picking your poison. That may well be true, ultimately preventing either family of superconductors from being widely usable. Along with osmium, arsenic, lead (which also forms good intermediary layers in cuprate superconductors) and beryllium, these are some of the most notorious poisonous elements that solid-state chemistry has to offer. I have always viewed the toxicity of the highest-temperature superconductors philosophically, believing that they

embodied on a microscopic scale the balance between good and evil in nature. In spite of the well known toxicity of mercury, one can find it in many everyday products such as thermometers, mercury-vapour lamps, dental preparations and mercury-cadmium batteries. The same cannot be said for thallium, which has been forbidden as a rat poison in the United States since 1975.

This week, when answering the usual "what's new?" question from an interested colleague, I answered that $\text{HgBa}_2\text{CuO}_{4+\delta}$ was new, with a T_c of 94 K. After a moment of silence and a deep, thoughtful look, he asked: "Haven't any of you guys gotten around to trying beryllium oxide yet?" □

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MOUSE GENETICS

YACs to the rescue

Mario R. Capecchi

ON pages 255¹ and 258² of this issue, Jakobovits *et al.* and Schedl *et al.* report the first successful transfers of yeast artificial chromosomes³ (YACs) carrying very long fragments of genomic DNA into the germlines of mice. In this context 'very long' means greater than 250,000 base pairs, and the technology will find many applications in the molecular analysis of the mouse.

For over a century geneticists interested in mammalian biology have focused their attention on the mouse. In many respects it is the ideal mammal — it is small, prolific and easy to maintain; and its generation time, three months, is remarkably short. Many inbred strains of mice of uniform genetic background have been developed, and more than a thousand mouse mutants have been identified and maintained over the past century. The approximate chromosomal location of many of these mutants is known, providing a linkage map containing more than one thousand entries. The vast majority of these mutants arose from serendipitous discovery of visible variations in form, behaviour or both. The task of isolating the gene responsible for any of these mutant defects is at present very labour intensive, which precludes any easy entry into the molecular analysis of the existing set of mouse mutants and so greatly limits the usefulness of this vast resource. The ability to introduce YACs into the mouse germline should help rectify this situation.

For most of the mutations, all that is known about the defective genes is their phenotypic effects and their approximate location on the mouse genetic map. The

first step in cloning these genes requires refining their chromosomal position relative to a dense genetic map. Development of such maps using either restriction fragment length polymorphisms (RFLPs) in interspecies crosses⁴ or simple sequence repeats (SSRs) in intraspecies crosses⁵ promises a significant reduction in the effort required to define the location of any gene of interest within a few million base pairs. The SSR mapping technique is particularly attractive because it is user friendly: the investigator can use crosses between common inbred strains of mice, and the points of reference on this genetic map, the SSRs, are readily available from catalogues of published PCR primer sequences that surround the mapped SSRs.

Experience with positional cloning of human disease genes, such as those involved in cystic fibrosis, neurofibromatosis or familial polyposis, shows that even when the position of a gene has been defined within one or two million base pairs and all the DNA sequences within this interval have been isolated, identifying the relevant gene can still be a formidable task. The difficulties are exacerbated in the mouse because most of these mutations are represented by only a single allele. Under these conditions, the classical method of identifying a gene by determining differences in the DNA sequence between mutant and wild-type alleles may not be possible.

It is in this context that the reports by Jakobovits *et al.* and Schedl *et al.* are particularly exciting. The potential now exists for researchers to use the YAC transfer technology to define the loca-

tion of a gene on a large DNA fragment by introducing YACs containing genomic fragments into mutant mice and scoring for the rescue of the mutant phenotype. Indeed, Schedl *et al.* were able to complement the albino phenotype by introducing a YAC carrying the mouse tyrosinase gene into the germline of albino mice.

Interestingly, the two groups used very different approaches, each having its potential advantages and disadvantages. Schedl *et al.* used standard transgenic technology that involved the microinjection of gel-purified YAC DNA into the pronuclei of mouse zygotes. The injected zygotes were then transferred into foster mothers to allow the embryos to come to term. In contrast, Jakobovits *et al.* introduced YAC DNA into mouse embryo-derived stem (ES) cells by fusion with yeast spheroplasts carrying the YAC. The recipient ES cells were *hprt*⁻, and the YAC carried human genomic sequences encompassing the *hprt* (hypoxanthine phosphoribosyltransferase) gene. Following the introduction of YAC DNA, Jakobovits *et al.* were able to select for *hprt*⁺ ES cells in culture. These cells were then used to generate germline chimaeras by microinjection of the cells into blastocysts and surgical transfer of the blastocysts into the uteri of foster mothers.

In both cases the YACs did not appear to suffer major DNA sequence rearrangements as a result of their transfer into the mouse germline. This is a remarkable feat. The evidence for the YACs being intact and functional in the mice of Schedl *et al.* is primarily biological. The authors generated animals containing one, two or four copies of the YAC, and the amount of tyrosinase RNA and protein synthesized in the transgenic mice was proportional to the YAC copy number present in the mice. Because the tyrosinase gene encompassed a large proportion of the genomic DNA incorporated into the YAC, and because each of the YACs appears to be functional, it is reasonable to suggest that these YACs are intact in these transgenic mice.

In the process of transferring the *hprt*-YAC into ES cells by yeast spheroplast fusion, Jakobovits *et al.* also transferred up to the entire yeast genome into the ES cells. To the degree that intactness of the yeast and human DNAs can be judged by Southern blot analysis using yeast *Ty* and human *Alu* repetitive DNA probes, respectively, the authors obtained cell lines and mice in which the introduced DNA appears to be largely intact. It is remarkable that the presence of the entire yeast genome in ES cells did not significantly perturb ES cell function either in culture or in the formation of the germline chimaeras.

The introduction of YACs into the mouse germline by direct microinjection of YAC DNA into pronuclei seems to be the simpler of the two procedures. Attention to experimental details was undoubtedly important in the success of Schedl *et al.* in transferring the 250-kilobase tyrosinase-YAC into the mouse germline without measurable rearrangements; whether this procedure will be limited to YACs small enough to be passed through the microinjection needle without shearing remains to be tested. The spheroplast fusion procedure should not be sensitive to the size of the YAC being transferred into the ES cells. However, more studies are needed to see whether the many yeast genes transferred with the YAC are expressed in the transgenic mouse and, if so, whether that influences mouse biology.

An additional report, on the transfer of a YAC carrying a human heavy chain immunoglobulin gene fragment into the mouse germline, will appear shortly⁶. The authors used yet a third approach, introducing the YAC into mouse ES cells by co-lipofection with an unlinked selectable marker. The co-transfer efficiency was not high; only 15 of over 1,000 clones contained the immunoglobulin gene, and of these only three appear to have the YAC largely intact. Human heavy-chain antibody was detected in the serum of transgenic offspring, showing the YAC to be functional. An advantage of using ES cells is that the cell clones can be fully characterized before generating the mice.

I have stressed the use of this technology for cloning genes in YACs by rescuing mouse mutant phenotypes, but there are many other applications. For example, the DNA elements that control the expression of a gene can be thousands of base pairs away from the sequences that encode the gene product, and introduction of very long DNA fragments into the mouse genome will increase the probability that the transgene will have all the DNA elements needed for its proper expression. Or large transfers could be used to explore long-range *cis* elements such as those controlling imprinting or X-inactivation. All in all, the new reports represent an exciting development for the molecular analysis of mammalian biology. □

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Something from nothing

C. J. Foot

A VACUUM is never absolutely empty — even with particles and light absent, interesting phenomena remain, the products of so-called vacuum fluctuations. That these electromagnetic fluctuations exist and have observable effects is an intriguing consequence of quantum electrodynamics (QED), the quantum field theory of electromagnetic interactions. The group of Ed Hinds at Yale University has succeeded in measuring the remarkably delicate force sodium atoms

and there is a ladder of evenly spaced energy levels. The level spacing corresponds to the energy of one photon so that, for example, the emission of a photon by an atom is simply a process in which the energy of a mode of the radiation field at the appropriate frequency is increased by one unit.

A fundamental result in quantum mechanics is that an oscillator with energy-level spacing $\hbar\omega$ ($\hbar$ is Planck's constant, ω , the oscillator frequency)

must have a minimum energy of $\frac{1}{2}\hbar\omega$ — the zero-point energy. This description of the radiation field in terms of a collection of harmonic oscillators gives a straightforward explanation of vacuum fluctuations as the fields associated with the zero-point motion of the oscillators. Even in the complete absence of real photons there is a finite energy in each mode of the field coming from the zero-point fluctuations.

The results of these fluctuations first became apparent in the 1940s with the measurement of the 'Lamb shift', a slight change in atomic energy levels attributable to their effect near a nucleus (these are akin to the spectroscopic Stark shifts induced by applied electric fields). What Hinds and colleagues have now measured is the more delicate force that arises when the vacuum fluctuations are modified by the proximity of a conducting plate (no electric field is applied).

Near a conducting plate, the number of modes of radiation field is reduced by the boundary condition that the electric field at the surface must be zero, and so the atomic energy decreases close to the conducting surface, which leads to an attractive force. This interaction between an atom and a conducting plane was first discussed by H. B. G. Casimir and D. Polder in 1948, but has never been measured before. The effects of the modified vacuum around a conducting plate can be probed using a second conducting plate instead of atoms. Casimir showed that the type of mechanism just described would also lead to a force between two such parallel plates in vacuum, and some qualitative observations of this Casimir force have been made.

The ideas have been tested much more stringently in the new experiment at Yale using the sensitivity afforded by atomic physics. To measure the Casimir–Polder force, atoms moving at thermal velocities were sent between two glass

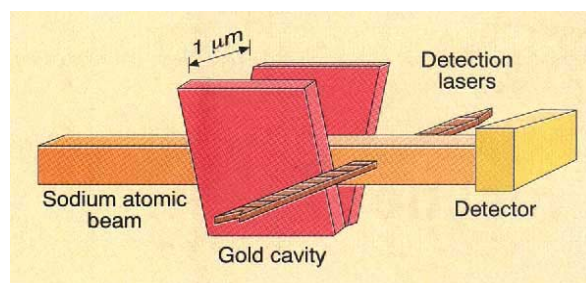


FIG. 1 To measure the Casimir–Polder effect, Hinds and colleagues passed sodium atoms through a wedge-shaped gap between two conducting sheets. The emerging atoms were detected by photoionization.

experience as a result of the QED fluctuations (C. I. Sukenik *et al.* *Phys. Rev. Lett.* **70**, 560–563; 1993).

To understand the physical origin of this force in more detail requires a closer look at the description of the radiation field in QED. It is well known that radiation is quantized into photons, but not the short, directed lengths of sinusoidal oscillation typically depicted in textbooks. Rather, it is better to think of radiation in free space as a superposition of many modes of oscillation within a box of arbitrary size. For a classical field, the energy in each mode would depend on the amplitude of the oscillations. According to QED, however, the radiation field of each mode is considered to be restricted to a set of discrete energy values. In fact, each mode behaves as a quantum harmonic oscillator,

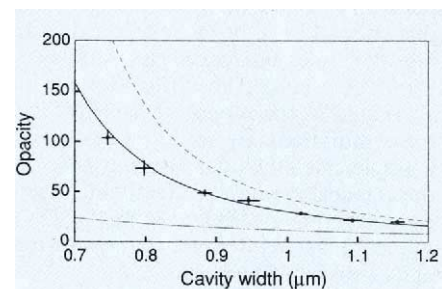


FIG. 2 Measured cavity opacity versus cavity width (error bars) and theoretical curves for van der Waals (top), QED (middle) and no (bottom) interaction.

plates coated in gold and separated by a spacer a few micrometres thick at the top (Fig. 1).

Hinds *et al.* measured the effect of different cavity spacings on the transmission of atoms through the gap by detecting atoms passing through the gap at different heights. Very efficient detection was achieved using a laser beam to ionize the emerging atoms. The physical separation of the gold surfaces at different heights was determined to within 5 nanometres by using monochromatic light to produce interference fringes — the number of half wavelengths at a given height was found by counting the number of fringes from the bottom where the surfaces were in contact. But the effective width of the gap for trans-

mission of atoms is smaller than the actual size because atoms near the walls are deflected. This has a significant effect on the fraction of transmitted atoms when the cavity spacing is around 1 μm . The reciprocal of the transmission of the gap, the opacity (see Fig. 2), shows clearly that there is an interaction between the atom beam and the plates, and the observations agree well with the behaviour expected for the Casimir-Polder force — there were no free parameters in this fitting. Sukenik *et al.*, it seems, have succeeded in bringing nothing into this world. $\square$

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MICROBIOLOGY

Giants among the prokaryotes

Mitchell L. Sogin

IMAGINE a world in which prokaryotes dwarf typical eukaryotes. A glimpse of such a world is provided on page 239 of this issue¹, where Angert and colleagues describe molecular data showing that symbionts that live in the gut of the surgeonfish, and which are some 80×600 micrometres (μm) in size, are closely related to Gram-positive eubacteria. This radical departure from 'normal' prokaryotic cell architecture underscores the point that many microorganisms that cannot be cultured in the laboratory may be stranger and more diverse than we realize.

Because of its enormous size, the surgeonfish symbiont *Epulopiscium fishelsoni* was originally described as a protist of unknown phylogenetic affinity². Then evidence that it is in fact a prokaryote of some sort came from electron microscopy studies³. Instead of nuclei, these giant symbionts contain bacterial-type nucleoids with no hint of surrounding membrane structure; and their flagella are bacterial-type rather than the classic 9+2 microtubule structures found in eukaryotic cilia. So gross morphology and ultrastructure point to different conclusions about the phylogenetic history of the organism, resolution of which is unlikely to be achieved by comparative studies of physiology and biochemistry because *E. fishelsoni* (as well as most symbionts and an untold variety of microbial species) cannot be propagated in the laboratory.

Modern molecular studies⁴ circumvent this difficulty by using standard cloning or polymerase chain reaction (PCR) methods to isolate genes that represent evolutionary homologues from natural populations. Comparisons with a large

database of similar sequences provide identifications of natural isolates. The small subunit ribosomal RNAs are particularly valuable for authenticating phylogenetic affinity and assessing the diversity of natural populations, and their widespread acceptance as 'molecular yardsticks' in phylogenetic studies has fuelled the rapid expansion of small subunit rRNA databases⁵.

Angert, Clements and Pace¹ took advantage of this framework to determine the proper phylogenetic place of symbionts harvested by micro-manipulation from gut contents of surgeonfish. Molecular trees unambiguously position *E. fishelsoni* with the low G+C Gram-positive bacteria. Carefully controlled *in situ* hybridizations with fluorescent rRNA probes confirmed that the symbionts were the source of the amplified sequences.

Now, consider some of the ideas that are affected by this humbling discovery of prokaryotic giants. Size is a frequently cited (but incorrect) criterion for differentiating between eukaryotes and prokaryotes⁶. Assumptions such as these colour our understanding of prokaryotic physiology and dominate theories about evolution into the primary lines of descent. Although cell volumes of eukaryotes are typically 100–1,000 times greater than those of prokaryotes, their range of sizes overlaps. For example, the chlorophyte *Nanochlorum eukaryotum* has a mitochondrion, a chloroplast and a nucleus contained within a cell 1–2 μm in size. Very long but skinny bacteria (greater than 200 μm by less than 0.75–8.0 μm) have been described^{7,8}, but they contain only nominal amounts of cytoplasm because of their spiral morphology

or the presence of large liquid vacuoles.

The $80 \times 600\text{-}\mu\text{m}$ cell dimensions of *E. fishelsoni* are far greater than the examples cited above, so the theoretical constraints on cell size imposed by prokaryotic cell architecture and apparent absence of intracellular vesicular transport mechanisms will have to be re-examined. Clements and Bullivant³ proposed that the peripheral layer of highly convoluted (plasma) membranes in *E. fishelsoni* solves the problem of transport to the interior and provides a large compartment for pooling protons to feed the battery of flagella required for motility. Mechanisms for faithful distribution of nucleoid structures (containing bacterial chromosomes) to daughter cells remain unclear.

Perceived size constraints for prokaryotes, and hence interpretations of microfossils according to size, have dominated traditional thinking about the evolutionary history of eukaryotes⁹. The evolution of large size is considered to be an automatic consequence of the evolution of the most fundamental eukaryotic characters, the endomembrane system and the cytoskeleton. The absence of large cells from the fossil record before 1.5–2.0 billion years ago is taken as evidence for the more recent appearance of cells with nuclei. But it now becomes clear that the assignment of eukaryotic status to microfossils is strictly an operational definition. The existence of small eukaryotes such as *Nanochlorum* and the discovery of giant prokaryotes lacking cytoskeletons and known vesicular transport system raises suspicions about the phylogenetic significance of size differences between eukaryotes and prokaryotes. This in turn will force re-examination of the fossil record as it pertains to the evolutionary origins of eukaryotes — indeed, molecular data¹⁰ in the form of rRNA sequence similarities are consistent with eukaryotic lineages diverging before three billion years ago. $\square$

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Magnetic geometry of sunspots

Nigel Weiss

SUNSPOTS, observed through telescopes since the time of Galileo, were shown to have magnetic fields by Hale at the beginning of this century. Although they seem ideally suited for theoretical study, they have proved extremely difficult to model in detail. Only now that their fine structure has been resolved can we see why. The white-light photograph below (*d*), taken on 2 July 1990 during exceptional seeing at the Swedish Solar Telescope on La Palma and processed at the Lockheed Palo Alto Research Labs, shows details of a sunspot's penumbral structure down to a scale of 200 kilometres (0.3 arcseconds). Title *et al.*¹ find that the directions of the magnetic field in bright and dark filaments are significantly different. At the outer edge of the spot, where the bright filaments are inclined at only 50° to the vertical, the dark filaments are almost horizontal

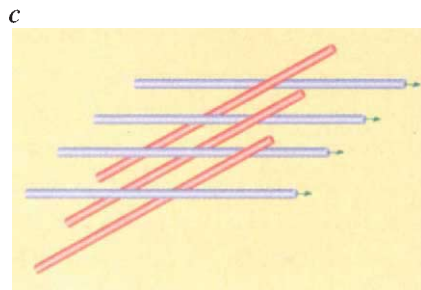
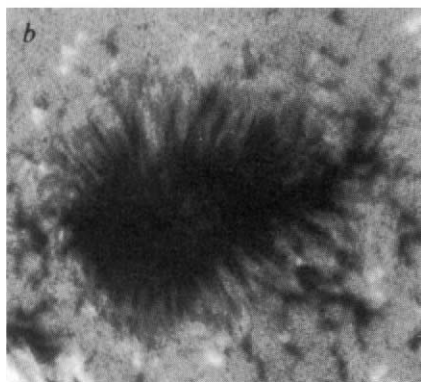
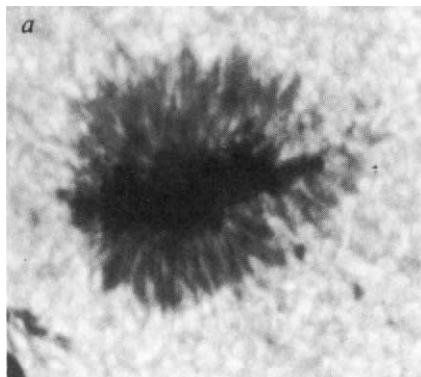
and aligned with a systematic radial outflow. This strange geometry affects the whole structure of a sunspot².

Sunspots are dark because the normal solar convective motion is suppressed by an intense magnetic field. The field can be measured by the Zeeman splitting of atomic spectral lines, and the plasma motion by their Doppler shift. At the centre of a sunspot, the magnetic field is vertical and has no significant azimuthal component. All observations indicate that the inclination of the field lines increases with radius. For many years there was disagreement as to the inclination at the outer boundary of the penumbra, but recent observations all concur in giving a mean inclination of about 70°. This was hard to reconcile with the large-scale radial outflow, first discovered by Evershed, which is horizontal and concentrated in dark fibrils, and

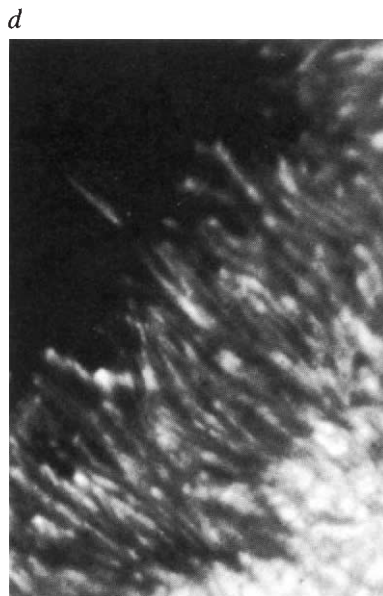
there were hints that the bright and dark filaments had different inclinations.

The new observations solve this problem. Precision measurements of spectral lines, using a narrow-band tunable filter developed at Lockheed, now make it possible to construct high-resolution magnetograms and Dopplergrams from which the line-of-sight components of the magnetic field and plasma velocity can be determined. Magnetograms obtained on La Palma show that the directions of the field in dark and bright filaments do indeed differ by 30–40°. At the edge of the spot, where the field in the dark filaments is almost horizontal, the inclinations are consistent with the observed mean field.

At the boundary between the umbra and penumbra, where the mean field is inclined at about 45°, the bright and dark filaments have fields inclined at about 30° and 60°, respectively. The field strength decreases outwards but does not vary significantly between bright and dark filaments at the same radius. Any concern that these results might be an



The two images to the left (*a*, *b*) show a sunspot observed with the Swedish Solar Telescope on La Palma on 7 June 1992 using the Lockheed tunable filter. The spot was near the centre of the solar disk and the images are 50 arcseconds (3,600 kilometres) across. The continuum image (*a*) shows the structure of the spot, with a dark umbra surrounded by a filamentary penumbra. The magnetogram (*b*) indicates the strength of the line-of-sight component of the magnetic field, which has different signs in regions that are black and white. Dark filaments in the continuum image correspond to regions where the field is almost horizontal and has virtually no component along the line of sight. *d*, Exceptional white-light image of the penumbra obtained in 1990. (Lockheed Palo Alto Research Labs.) The sketch (*c*) illustrates the interlocking comb-like structure of the field in the outer part of the penumbra. Magnetic flux tubes in the dark filaments (blue) are almost horizontal, whereas those in the bright filaments (red) are inclined. The radial outflow (green) is confined to the dark filaments. The X-ray image (*e*), obtained with the Normal Incidence X-ray Telescope on 22 February 1991, is superposed exactly on a white-light image. Magnetic loops emerging from the penumbra of the prominent sunspot can be followed for distances comparable with the solar radius. (IBM Research and Smithsonian Astrophysical Observatory.)



artefact, caused by the use of a birefringent filter instead of producing spectra with a grating, was removed when filtergrams and spectra were obtained simultaneously at the German Vacuum Tower Telescope on Tenerife³. The Evershed flow turns out to be intermittent, with puffs that progress outwards along adjacent dark filaments; their proper motion agrees with the measured Doppler velocities of 1–4 km s⁻¹.

But how is it possible for the light and dark filaments to have such different field inclinations, giving this fine, interlocking comb-like structure? And how is this pattern related to energy transport by convection? Spots that are sufficiently small (pores) lack a penumbra; also it seems clear that with greater magnetic flux, the field at the outer edge becomes increasingly inclined until it becomes unstable to perturbations with a high azimuthal wavenumber, which develop to produce a filamentary penumbra. Filaments alternate between being dark and bright, picking up heat first and then radiating it, so providing a mechanism for transporting energy through the penumbra to the surface^{4,5}. Not all of the bright filaments can take part, however, for some are connected to spectacular magnetic loops which extend for many spot radii across the solar surface⁶. Numerical experiments have helped to provide an understanding of magnetoconvection in the umbra⁷, where the mean field is roughly vertical, but it is far more difficult to model the penumbra, where the geometry is awkward and the boundary conditions are uncertain. Yet it is the mean stratification of the penumbra that determines the overall structure of a sunspot in magnetohydrostatic equilibrium, for the magnetic field at the centre is not strong enough to support the spot against external pressure⁸.

The filamentary penumbra also raises questions about the global structure of sunspots and starspots. Is there a more or less coherent magnetic plug extending deep into the convection zone or does it split into a cluster of isolated flux tubes? Can such a cluster be held together

by hydrodynamic interactions⁹? Theory may not succeed in answering such questions on its own but helioseismology offers a means of probing deeper structure. The existence in a sunspot of acoustic *p*-modes with periods around 5 minutes confirms that the flux tube must be coherent down to depths of several thousand kilometres¹⁰. Recent observations show that spots absorb incident

p-modes from the surrounding photosphere^{11,12} and we may expect that in the future more precise measurements will reveal the structure of the flux tube where it cannot be observed directly. □

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PALAEONTOLOGY

Graptolites come to life

Sue Rigby

ALL palaeontologists dream of finding a 'living fossil'. Noel Dilly, it seems, has done so and an account of the discovery appears in a recent issue of the *Journal of Zoology*¹. A trawl from deep water off New Caledonia, half way between Brisbane and Fiji, has brought to light an extant pterobranch (a colony-forming hemichordate) that has an astonishing physical resemblance to graptolites, a group considered to have been extinct since the Carboniferous, 300 million years ago. What is most significant is that the new species, dubbed *Cephalodiscus graptolitoideus*, carries on its skeleton long spinose structures very like those that have been a source of controversy in graptolites for almost 30 years. With the new discovery those arguments can be laid to rest.

The closeness of relationship between pterobranchs and graptolites has been the subject of much debate. As graptolites are arguably the most important zone fossils of the Lower Palaeozoic (570–360 million years before present), this is far from being an esoteric issue. Only the hard parts can be compared, but fortunately they are grown by living pterobranchs in a characteristic fashion. Collagen is the main skeletal element and it is plastered onto the colony skeleton, the coenecium, with a sucker-like cephalic shield. Crowther² showed that most of the graptolite periderm was probably also constructed in this way — graptolites grew increments to their colonies and plastered narrow bandages on top, which look tantalizingly like the work of a cephalic shield.

But the general view is that one part of the graptolite skeleton could not have been secreted in this manner. This is the

nema — a long spine which extends for many times the length of a zooid and which is common to most planktonic graptolites (Fig. 1). How could a graptolite zooid, attached to others in the colony by soft tissue, have left its theca and climbed for many times its own length to secrete material at the tip of the nema? The question seemed all the

more difficult to answer in many later graptolite species in which the aperture of each theca is constricted. It has long been assumed that the zooid would thereby have been imprisoned in its thecal 'cell' and unable to venture out across the colony surface. It is impossible to resolve this problem by reference to the fossil record alone.

If the nema was not constructed by the zooid, it must have been produced by other means. But how? Suggestions have included secretion by an extra-

thecal membrane completely covering the colony³ or from blobs of soft tissue at the nema tip⁴. The 'nema problem' has become the single most powerful argument against a relationship between graptolites and pterobranchs — if the method of secretion of the living group fails to explain the secretion of part of the fossil, then the main argument for proposing such a relationship is invalid.

Hence the importance of *Cephalodiscus graptolitoideus*. The spines of this pterobranch are up to 35 mm long, yet the zooids never exceed 1.5 mm in length; moreover, the coenecial apertures are constricted, and have a smaller diameter than do the zooids. It turns out that when a spine is under construction, the zooids squeeze out of their colony, crawl up to 30 times their own length, secrete an increment of spine and return

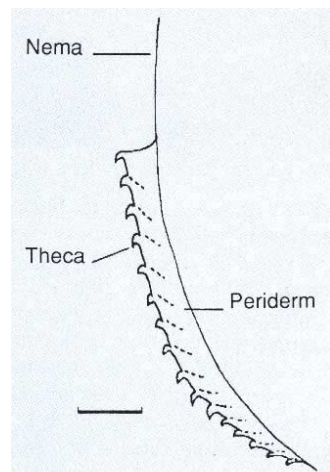


FIG. 1 The main morphological features of graptolites. Scale bar, 2 mm.

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through their narrow apertures to their 'cells'. The spines are used in feeding, to elevate the zooids above the slow-moving boundary layer of water close to the shells on which they live (Fig. 2).

Other living pterobranchs grow small spines in a similar way, but the discovery of such long processes is unique. It elegantly and unequivocally removes the difficulty in explaining how pterobranch-like zooids could have secreted the

by *C. graptolitoideus* than there are zooids in the colony and there is clearly a degree of cooperation between zooids. There is no rigid colony architecture but the zooids work together to build functional units. It should be possible to determine the controls on this interaction. In contrast, the planktonic graptolites were built with a high degree of regularity and symmetry. The number of thecae, the stipe width, the degree of



FIG. 2 *Cephalodiscus gracilis* feeding on a transparent spine.

nema. To all intents and purposes, graptolites and living pterobranchs can be regarded as a single phylogenetic group. Whether *Cephalodiscus* is a living genus of graptolite, as Dilly suggests, or whether graptolites are an extinct group of pterobranchs, remains to be decided; on balance, many characteristics of extant pterobranchs seem primitive compared with the highly specialized morphologies seen in graptoloids⁵. These taxonomic differences are, however, largely a matter of terminology. What is important is the undoubted closeness of relationship between the two groups.

Palaeontologists can now reconsider the functional morphology of graptolites afresh, no longer plagued by doubts about their affinity. Study of living pterobranchs can be extended in the knowledge that their comparison with graptolites will yield new insights about the extinct forms. It becomes possible, for instance, to use coenecium growth rates of extant pterobranchs to calculate a minimum lifespan for graptolites because we are now sure that they built their colonies in much the same way. Population studies will be possible for the many bedding planes where graptolites are preserved abundantly and as the victims of mass mortality.

Another area open to investigation is the relationship of the individual zooid to the colony. Fewer spines are secreted

overlap between thecae and the design of thecal ornamentation all show consistent variation along the length of the colony. The observation of loosely organized cooperation in living, benthic pterobranchs implies that the unusually tight organization of graptoloids was determined largely by their planktonic mode of life, as this is the main remaining difference between the two groups.

A simple but valuable lesson emerges from the discovery and analysis of *C. graptolitoideus*. The biology of most fossil organisms is best studied with reference to the living world, yet collaboration between zoologists and palaeontologists remains patchy at best. The advantages of such mutual awareness are amply demonstrated by Dilly's study; he was sufficiently familiar with a major fossil group to realize the importance of a new zoological find. The alternative can be years of fruitless wrangling and the stagnation of whole fields of study. □

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DAEDALUS

Sun and shadow

AMONG the underpigmented peoples of the world, the craze for a chic sun-tan is still alive. A sun-tan, of course, is the body's defence against the ultraviolet component of direct sunlight. These days, with the thinning of the ozone layer, solar ultraviolet is beginning to overwhelm this defence. The Sun lover now risks getting skin cancer. But while this cancer can take many years to develop, the perceived benefits of a tan are immediate and powerful. They include enhanced social popularity and sexual desirability, leading to a wider choice of mates and therefore a greater likelihood of high-quality offspring. Those who go for the tan and risk the cancer are probably acting in their own best evolutionary interests.

Tanning, says Daedalus, also has another biological benefit. It is not a mere uniform coloration. It preferentially darkens the sunlit areas of the skin. It thus acts as a self-optimizing camouflage, reducing the body's optical contrast. (Many animals are darker on the top and lighter on the underside for just this reason.) Our primitive ancestors, anxious not to attract the attention either of fierce predators or alert prey, would have valued a sun-tan for added obscurity rather than conspicuous chic.

So Daedalus wants to make tanning safer. He remarks that the first silver-halide photographic emulsions were sensitive only to ultraviolet and blue light. Early photographers used ultraviolet-rich light sources, such as direct sunlight or the rather fearsome explosion of carbon disulphide vapour in nitric oxide. But then photographic sensitizers came along: dyes that absorb visible light and hand its energy to the silver halide. The resulting panchromatic film was sensitive to the whole visible spectrum.

The same trick might work on human skin. Daedalus is coating DREADCO volunteers with a variety of dyes, from photographic sensitizers to traditional war-paints, and is then exposing them to light of different wavelengths. He hopes to discover a skin pigment that will activate a tan even in overcast British daylight, perhaps even under translucent clothing. When in due course the user washes off the dye, a chic, safe, becoming tan will be revealed.

Daedalus has particularly high hopes for that ancient blue skin dye, woad. He suspects that the ancient Britons (who had quite as much trouble getting sun-tanned as their modern counterparts) used it not merely as a terrifying war-paint, but as an activator for a camouflaging tan. It served a double duty, attracting attention and fear when worn, and deflecting them when washed off.

David Jones

Population size and viability

SIR — Decimation and fragmentation of plant communities are a threat to the maintenance of biodiversity. As a result of genetic erosion or reduced availability of suitable pollen¹, smaller populations can be expected to produce disproportionately fewer seeds until a critical population size is reached, beyond which seed production may be negligible and extinction inevitable (the Allee effect²).

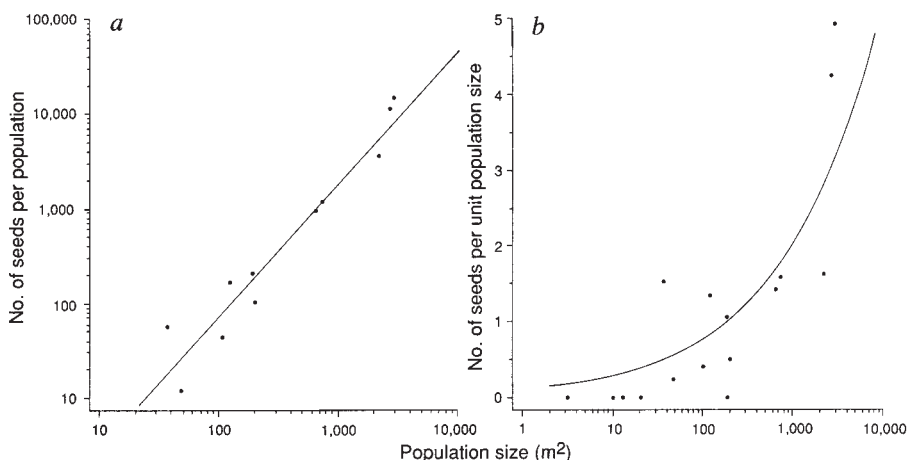
Banksia is an important genus of 75 species in the vegetation of southern Australia. Some species are rare and on the verge of extinction. *Banksias* produce infructescences (cones) which bear follicles, each containing two seeds. Seeds are retained until the follicles open in response to fire or the cones begin to disintegrate after ten or more years. *B. goodii* is a woody prostrate species pollinated by mammals (honey possums) and birds (honeyeaters). Agriculture and road building have reduced the abundance of *B. goodii* to 16 known populations occurring in a $37 \times 13 \text{ km}^2$ area north of Albany, Western Australia ($34^\circ 45' \text{ S}$, $117^\circ 40' \text{ E}$). Nine populations are small remnants on road verges.

We assessed all individuals of all known populations for seed production in September–October 1992. Small populations produced none or only a few seeds per unit canopy area (see figure). Effects of population size on seed production per unit area were present in the whole range of population sizes (b in the figure), indicating that even in the large populations seed production may still not be at its maximum. Resource differ-

ences could not explain this disproportionate decrease in seed production with decline in population size because there were no differences in soil properties and understorey or overstorey cover between the small and large populations. Although plants in small and large populations are similar in size, seed production per plant is much lower in small populations. This is not because plants in small populations produce fewer cones but because the fraction of fertile cones is much lower. Five of the nine smallest populations ($<200 \text{ m}^2$) produced no fertile cones over the past 10 years. The number of seeds per fertile cone does not depend on population size.

The reduction in the fraction of cones bearing seeds among smaller populations indicates the importance of pollen quality and/or quantity. Among banksias, seed set is rare or zero in the absence of pollinators³ and high levels of outcrossing are the norm⁴. The loss of birds from fragmented habitats is well known⁵. In addition to the reduced level of pollination this may cause, pollen transfer may increasingly be between siblings as population size is reduced.

Attitudes to the value of small populations in conserving rare species vary^{6,7}. In *B. goodii*, five of the small populations lacked any seeds and two had far fewer seeds per unit canopy area than the larger populations. Together with their small population sizes, the contribution of these remnants to the conservation status of *B. goodii* must be



a, Total seed production per population (Y) decreases more than proportionally with decline in population size (X). **b**, In small populations seed production per unit canopy area is lower than in larger populations. Population size is gauged as $\Sigma(\pi/4)D_1D_2C$, where D_1 is maximum diameter, D_2 that at right angles, and C projective canopy cover. All but one population had not been disturbed for at least 20 years (no fires or soil disturbance) and all cones <10 years old were intact and retained all their seeds. To test for proportionality in **a** we used the model $Y = aX^b$, or $\log Y = a' + b \log X$ (ref. 8). After omitting populations with no seeds we obtained: $\log Y = -0.99 + 1.43 \log X$; $R^2 = 0.93$, $P < 0.0001$; test on $H_0: b = 1$, $H_1: b > 1$: $F = 11.35$, $P = 0.004$). To avoid spurious correlations the line in **b** is based on the regression equation in **a**. In **b**, populations with no seed production have been added.

considered negligible. The sobering message is that a series of small populations may not have the same conservation value as a larger one with the same total population size, while there may be a threshold below which local extinction is inevitable.

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ATP in synapses

SIR — Although we agree with McLachlan and Keast¹ that “it would be unfortunate if the idea that ATP is commonly a cotransmitter at cholinergic synapses entered the textbooks”, we think it would be equally unfortunate if their comments leave the impression that ATP cannot yet be accepted as a neurotransmitter in the peripheral or central nervous systems.

As pointed out by Benham², the evidence is now overwhelming that ATP is a major^{3,4} vasoconstrictor substance released from noradrenergic sympathetic nerve terminals onto blood vessels. We⁵ and Edwards *et al.*⁶ have demonstrated that ATP can also mediate fast ligand-gated synaptic transmission at nerve-nerve synapses. Edwards *et al.* did not suggest that the ATP synaptic current they identified in slices of the medial habenulum derived from the known cholinergic input to this brain region, but there is no evidence against this (or any other) presynaptic origin.

We recorded ATP synaptic currents from noradrenergic coeliac ganglion neurons maintained in tissue culture and thus deprived of both their preganglionic cholinergic input and their postganglionic vascular targets. McLachlan and Keast find this result “unexpected” because only nicotinic fast synaptic potentials have been recorded from intact coeliac ganglia and because previous studies on other sympathetic ganglia showed that many noradrenergic

neurons become cholinergic when placed in tissue culture^{1,7}. But this adrenergic-to-cholinergic switch is triggered by conditioned medium most easily in neurons from neonates and occurs earlier rather than later during the culturing process⁷⁻⁹. The adult coeliac neurons

we^{5,10} and others¹¹ have studied are not provided with conditioned medium and maintain their catecholaminergic phenotype for more than 2 months in culture. Nicotinic synaptic transmission therefore is not to be expected and has not been observed; only ATP fast synaptic potentials have been recorded during this time. McLachlan and Keast appear to disdian these purinergic synapses as an artefact of culture; we view such synaptic purity as a virtue rather than a vice for studying mechanisms of action of this 'new' fast neurotransmitter.

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HIV vaccination dilemma

SIR — Ada *et al.* in their Commentary¹ discussed my paper² on the improbability of effective vaccination against human immunodeficiency virus (HIV) because of its intracellular transmission and rectal (or vaginal cervical) port of entry.

Neither antibodies nor cell-mediated immunity (CMI) is effective against intracellular transmission of HIV infection — CMI is directed against the viral antigens expressed on some HIV-infected cells but not on others in which HIV is suppressed as complementary DNA of HIV integrated in chromosomes. Ada *et al.* pointed out that such cells are, as a rule, foreign (allogeneic) to the recipient. Accordingly, they assumed that such allogeneic HIV-infected lymphocytes in the semen (or blood) would be rapidly destroyed by the recipient host cells, and that "if the HIV cDNA in live or dead semen cells were to be transferred undamaged in some novel way to the live host cells, as Sabin suggests, expression of viral peptides could also occur. If so, the 'transfected' host cells would be recognized by cytotoxic T lymphocytes (CTLs) in the vaccinated host." They added that "Cells in the donated semen already expressing viral antigen could fuse with host cells (gp120/CD4 interaction), and thus be recognized by both class I MHC restricted CTLs resulting from the vaccination and alloreactive CTLs."

The 'flaw' in this argument is that there is a "novel way" by which HIV-infected lymphocytes have been demonstrated to be capable of rapidly transferring infectious virus, either HIV RNA or chromosomally integrated HIV cDNA (without expressed viral antigens or peptides combined with class I MHC), in HIV-infected cells by cell-to-cell contact without using CD4 receptors. This phe-

nomenon was recently described by Phillips and Bourinbaier³, who showed that "contrary to infection with free virus, the cell to cell infection was not blocked by anti-gp120 or antiviral serum from HIV-positive individuals". These authors concluded that these "observations offer an insight into the cellular sequence of events which may take place during sexual transmission of HIV across an intact epithelial barrier." Even before HIV was identified as the cause of AIDS, Shearer⁴ suggested "allogeneic leukocytes as possible factors in induction of AIDS in homosexual men". Ada *et al.*, in downplaying "the efficacy of transmission of HIV infection by sexual intercourse, including receptive anal intercourse, and needle sharing" disregard the fact that in the United States in 1991, 64% of the reported cases of AIDS occurred in homosexuals, 6% in heterosexuals, and 23% among people involved only in intravenous drug abuse. The reported rapid transfer of HIV in its various intracellular states by cell-to-cell contact³, before the allogeneic mechanism can come into play, is the "novel way".

Also of relevance is the report⁵ that purified proviral DNA of simian immunodeficiency virus (SIV) is infectious *in vivo* but not *in vitro*. Ada *et al.*, in pointing to the effectiveness of some vaccines against intravenous challenge by cell-free virus, also overlook a report⁶ showing that vaccinated monkeys protected against an intravenous challenge with 25 monkey-infectious doses of cell-free SIV were not protected against a similar dose of intracellular SIV.

It is evident from the natural history of HIV infection in human beings that the antibody and cell-mediated immune responses are inadequate in preventing the accumulation in the lymph nodes and

other tissues of a reservoir of HIV-infected cells (many with only chromosomally integrated suppressed cDNA), which remain dormant for many years until they are activated (derepressed) by as yet incompletely understood events. Moreover, there is increasing recognition of mechanisms by which host immune defences are counteracted to produce latent and persistent infections by viruses⁷.

The continuing failure to use infected cells by various routes, rather than only cell-free virus by the intravenous route, to test for protection produced by vaccination was again evident in a report⁸ on the protective effect of a live attenuated SIV vaccine, with a deletion of the *nef* gene, in which two of four rhesus monkeys which previously resisted an intravenous challenge with ten cell-free monkey-infectious doses of virus, resisted a subsequent intravenous challenge with 1,000 cell-free monkey-infectious doses. The enthusiastic response of HIV vaccinologists to this finding⁹ shows the widespread naivety about what live virus vaccines cannot be expected to achieve. I said² that: "A live genetically engineered safe deletion mutant of HIV or SIV cannot be expected to do more than [natural infection with] the unmodified virus", which may perhaps be called Sabin's law about live virus vaccines.

There is no scientific evidence that any of the experimental vaccines against SIV or HIV, some of which are scheduled for use in large-scale human trials, have any protective effect against natural modes of transmission in previously uninfected or latently infected hosts. Ada *et al.* said that "to delay or not perform such trials for the reason proposed by Dr Sabin would be disastrous for the increasing numbers of people exposed to the risk of infection". In my judgement, it would be disastrous to continue the current inadequate methods of study of HIV and SIV vaccines, and to carry out large-scale tests in humans of vaccines without adequate evidence that such vaccines can protect against natural infection with adequate doses of intracellular virus.

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* Dr Sabin died on 3 March. *Nature* will be printing an obituary shortly.

Ferroelectricity origins

SIR — Bussmann-Holder and Büttner¹ make two remarks about my work² on the origin of ferroelectricity in perovskite oxides: (1) that the results of self-consistent electronic structure calculations are not unique and neglect dynamical properties; and (2) that the venerable shell model³, in which one fits numerous empirical parameters to experimental data, is sufficient to understand ferroelectrics.

The only input to modern electronic structure computations such as those I presented² are the nuclear charges and the positions of the nuclei, the latter of which are varied to map out the dynamical potential surface. The computations use no adjustable parameters, and thus are unique. In principle, such frozen phonon calculations allow the computation of any desired harmonic or anharmonic dynamical property, though owing to tremendous computational demands, I presented only the potential surface for the ferroelectric soft mode for nuclear displacements with a periodicity of the primitive cubic perovskite cell². The finite temperature dynamics is not neglected but is investigated by parametrizing the potential surface obtained from first-principles calculations^{4,5} rather than by experiment. The dynamics of the phase transition was not the subject of my paper², which was about the origin of the ferroelectric instability.

The computations show incontrovertibly that the ferroelectric instability is not due to shell-model-like polarizability of the oxygen ion as advocated by Bussmann-Holder and Büttner. The shell model is an empirical model in which parameters are fitted to experiment, and there is no guarantee, as with any empirical model, that the fitting parameters have any physical meaning, or that the model correctly describes the underlying physics behind material behaviour. The present computations clearly show the importance of covalency in driving the ferroelectric instability, and illustrate the advantages of first-principles methods compared with parametrized models.

In the 30 years since the shell model was proposed, there have been many attempts to model ferroelectrics and other phase transitions. Although im-

pressive agreement with experiment is sometimes obtained by introducing sufficient parameters, the ability to predict unknown properties of materials, or to predict properties of new materials, has not been demonstrated. First-principles approaches now give us the ability to predict and study new materials and to understand the underlying physical processes that govern material behaviour.

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An anti-prion protein?

SIR — The physiological function of the prion protein (PrP) is unknown, but there is strong evidence that the infectious agent of transmissible spongiform encephalopathies (the prion) consists at least in part of PrP^{Sc}, a modified form of the normally occurring cellular PrP (PrP^C). Both forms of PrP are encoded in a single exon of the PrP gene^{2,3}. Goldgaber in his Scientific Correspondence⁴ noted a large open reading frame (ORF) on the antisense strand of the coding regions of mammalian PrP genes. Several genetic traits influencing spongiform encephalopathies in humans and animals have been linked to the PrP gene¹. If an 'anti-PrP' existed it could be responsible for the properties formerly ascribed to the prion protein⁴. In support of this hypothesis, Hewinson and colleagues detected a 4.5-kilobase RNA in bovine brain which hybridized to PrP sense riboprobes, and which was thus expected to represent a PrP antisense transcript⁵. We have found a similar 4.5-kb mRNA in normal and scrapie-infected hamsters and in mice; however, our results show that this presumed 'anti-prion protein' RNA is not derived from the antisense strand of the PrP gene.

We used the sequence of the second exon of the hamster PrP gene to generate sense riboprobes (Fig. 1a). Riboprobe A detected a 4.5-kb 'antisense' RNA in brains of normal and scrapie-infected hamsters at approximately the same level, as judged by northern analysis (Fig. 1b). The 4.5-kb RNA was polyadenylated and was not found in tissues other than brain (Fig. 1b). The fact that the 4.5-kb RNA was detected under stringent hybridization conditions initially suggested that the 'anti-PrP' RNA was transcribed from the opposite strand of the hamster PrP gene.

If indeed the 4.5-kb RNA were an antisense transcript of the PrP gene, then it should be absent or at least altered in mice homozygous for partially

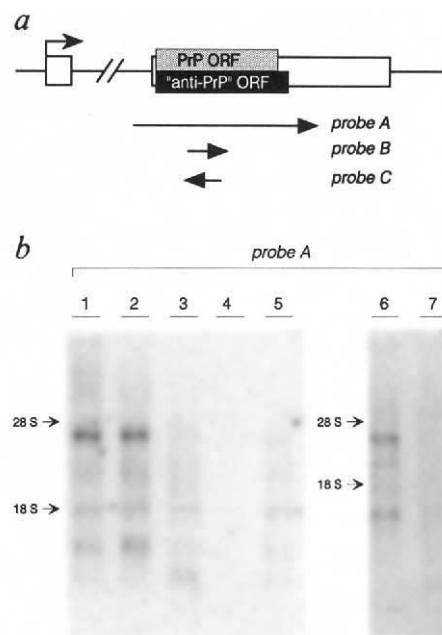


FIG. 1a, Genomic map of the hamster PrP gene³. Digoxigenin-UTP-labelled riboprobes A–C were generated *in vitro*; arrows denote direction of transcription. Probe A corresponds to the *EcoRI* to *DraI*; probe B to the *NcoI*–*HincII* fragment of the hamster PrP gene³; probe C corresponds to the *KpnI*–*BstEII* fragment of mouse PrP (ref. 11). b, Northern blots of total brain RNA from normal (lane 1) or scrapie-infected (lane 2) hamsters. Lanes 3–5 show spleen, liver and lung RNA, respectively, from normal hamsters. The 4.5-kb RNA is specifically detected in polyadenylated (lane 6) but not in non-polyadenylated (lane 7) RNA from normal hamster brain. Each lane contains 4 μ g total RNA, 4 μ g non-polyadenylated RNA or 0.4 μ g polyadenylated RNA. Hybridization was done with 50–100 ng probe per ml in 50% formamide, 5xSSC, 2% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 0.02% SDS overnight at 68 °C. Hybridized probes were visualized using chemiluminescence. Exposure times: lanes 1–5, 25 min; lanes 6, 7, 15 min.

deleted PrP genes (Prn-p^{0/0})⁶. Using the above hamster probe A, which was shown to cross-hybridize to 'anti-PrP' RNA from wild-type mouse brain (Fig. 2, lanes 1 and 2), we detected the 4.5-kb RNA also in mice with Prn-p^{0/0} genes (Fig. 2, lane 3). Because probe A extended beyond the partial deletion in the PrP gene of Prn-p^{0/0} mice, we used an additional probe which lies entirely within the deleted region (probe B). It detected the same 4.5-kb RNA in both types of mice (Fig. 2, lanes 4 and 5). These results unequivocally show that the 4.5-kb RNA is not derived from the opposite strand of the murine PrP gene; however, it appears to share considerable homology with the antisense strand of the PrP gene.

Our results confirm the presence of the 4.5-kb 'anti-PrP' RNA in two additional species, show that its concentra-

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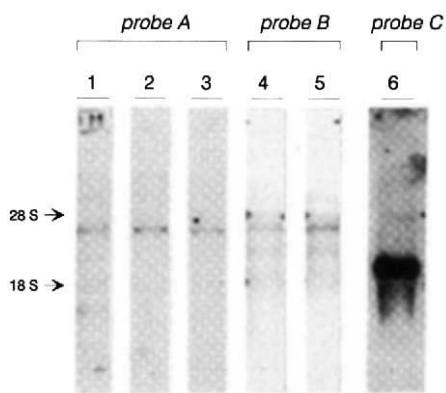


FIG. 2 Northern blots of brain RNA from hamster (lanes 1, 6), wild-type mice (lanes 2, 4) and mice homozygous for ablated PrP genes (*Prn-p^{0/0}*; lanes 3, 5). The 4.5-kb RNA species is detected in brain tissues of both wild-type and *Prn-p^{0/0}* mice. Sense probes A (lanes 1-3) and B (lanes 4 and 5) were used to detect the 4.5-kb RNA, antisense probe C (lane 6) detected PrP mRNA. Northern analyses were done as described in Fig. 1, with total hamster or mouse RNA (3 µg per lane) and polyadenylated mouse RNA (0.6 µg, lane 4, 1.2 µg, lane 5). Exposure times: lanes 1-3, 6, 15 min; lanes 4, 5, 9 min.

tion is not greatly altered after scrapie infection, and demonstrate that the RNA is not derived from the PrP gene. If the 4.5-kb mRNA, despite its being

derived from a locus other than the PrP gene, encoded an 'anti-prion protein', this might have intriguing implications. It has recently been described for four independent proteins that peptides encoded by antisense sequences correspond structurally to binding proteins or receptors⁷⁻¹⁰. Thus, a putative 'anti-prion protein' may be pertinent to the normal function of the prion protein.

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cannibalism by the surface markings on cranial and post-cranial bones, the degree of fragmentation, and by the presence of bones among kitchen refuse. Others have disputed the reliability of the original excavation work and evidence-gathering techniques, attributing the breakage to sediment pressure or rockfall and some of the presumed cutmarks to damage during bone recovery⁶. Cutmarks that could not be disputed were attributed, on this view, to non-cannibalistic ritual practice.

At the root of the controversy lie the different interpretations given to certain types of bone fractures. Evidence from ethnographic studies of mortuary practices involving bone defleshing and secondary burial shows that the bones are gathered with great care and never broken up^{7,8}. Consequently, it is very difficult to attribute fresh bone breakage accompanied by cutmarks to a simple mortuary ritual. Some of the objections raised in connection with the Krapina bones are not valid for the Abri Moula material because the bone fragments were found apart in undisturbed levels containing few rocks. The great interest of this new discovery is highlighted by the presence of a fresh bone breakage pattern (V-shaped and bevelled break pattern at an oblique angle) associated with cutmarks. The same modifications can be observed on faunal remains mixed with human bones.

Although our new osteological data cannot prove the practice of cannibalism among the Neanderthals, they constitute an important argument in favour of the hypothesis that such practices could have occurred among the Middle Palaeolithic inhabitants of Western Europe.

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Cannibals among the Neanderthals?

SIR — The question of prehistoric cannibalistic behaviour is a hotly debated issue¹⁻³. Recently, 13 Neanderthal remains were discovered by one of us (A. D.) at a depth of more than 6 metres in a small excavation in the Abri Moula, near Valence, southeast France. The biostratigraphic context belongs to a temperate period. We discovered human bones among lithic artefacts, defined as typical Mousterian, and faunal remains. There were four bones in level C, eight in level D and one in level E. Radiocarbon dates for charcoal from level C gave an age in excess of 49 kyr. The

initial palaeoanthropological identification showed seven fragments of cranial vault, three teeth and three postcranial fragments. Although none of the osteological fragments was redundant, the disparities in the robustness of several pieces suggest that more than one individual is represented, a conclusion consistent with the fact that the remains come from two distinct levels.

We could not determine how old the individuals were at the time of death, but a relatively young age is suggested by the slenderness of several fragments. The presence of several Neanderthal

autapomorphies is fully consistent with the archaeological context. Four of these remains (three skull fragments and a proximal radius epiphysis; see figure) bear clear cutmarks, undoubtedly resulting from a flint tool, associated with signs of fresh bone breakage. Cutmarks on postcranial Mousterian human remains have, until now, been observed only at the sites of Krapina⁴ and of Combe-Grenal⁵.

Some researchers define the existence of prehistoric



Radius showing clear cutmarks on the bicipital tuberosity (× 0.9).

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Aero-internationalist

Kenneth Owen

The Universal Man: Theodore von Kármán's Life in Aeronautics. By Michael Gorn. Smithsonian Institution Press: 1992. Pp. 202. \$24.95, £19.50.

FOR Theodore von Kármán, the sight of Henry Farman flying a Voisin biplane at Issy-les-Molineux on the outskirts of Paris on 21 March 1908 lit a fire of enthusiasm for aeronautics that was to burn brightly for more than half a century. Best known in his later years as head of NATO's Advisory Group for Aeronautical [later "Aerospace"] Research and Development (AGARD), the Hungarian-born mathematician and physicist forged a remarkable, multifaceted career in Europe and in his adopted country, the United States.

Acknowledged and celebrated internationally by his peers, von Kármán's influential contributions to aerodynamic theory need no repetition today, nor does Michael Gorn attempt this. His book sketches the main outline of his subject's life with a broad but perceptive brush. It is a complex picture, mixing theory and application, teaching and conviviality, astute lobbying and the search for scientific truth.

Born in Budapest in 1881, von Kármán studied engineering in Budapest, followed by graduate study in mechanics at Göttingen University under Ludwig Prandtl. His contributions to fluid dynamics included the explanation of 'vortex sheets', which extended the understanding of flow based on Prandtl's boundary-layer theory; his contribution to Germany's aeronautical science included building up the resources and reputation of the Aachen Technical University and Aerodynamics Institute, and applying these resources to Junkers aircraft and Zeppelin airships. By the early 1920s his international reputation was secure.

But the key event in von Kármán's career was his move from Europe to the United States in 1930, at the age of 49. It took Robert Millikan of the California Institute of Technology at Pasadena and philanthropist Harry Guggenheim four years to seal the deal, offering the Hungarian two directorships — of the Guggenheim Aeronautical Laboratory at Caltech (GALCIT) and the Daniel Guggenheim Airship Institute.

At the Guggenheim Laboratory, work

on aeronautical structures and aerodynamics fed into the designs of Douglas, Lockheed, Boeing and other west-coast companies. After an abortive attempt to collaborate on rocket propulsion with Robert Goddard, von Kármán established a rocket team at GALCIT. The



From *The Universal Man*

Von Kármán (1881–1963): a pioneer of supersonic flight.

team's work led to 'jet-assisted take-off' aircraft experiments using solid-propellant rocket units and to the formation of Aerojet General Corporation. In 1944 the GALCIT team was expanded to become the Jet Propulsion Laboratory.

During the Second World War, in addition to many other commitments, von Kármán was asked to form a group of leading aeronautical scientists to advise the Pentagon on airforce research and development. Thus the Army Air Forces Scientific Advisory Group was born. It took determined lobbying, not to say conspiracy, to ensure that the

new, postwar US Air Force set up a strong, continuing research and development organization that included von Kármán's group.

It took even more determined lobbying for von Kármán to implement his greatest single achievement — to mount a European equivalent of his advisory group as part of the newly formed North Atlantic Treaty Organization. The resulting NATO Advisory Group for Aeronautical Research and Development, AGARD, opened for business in the Palais de Chaillot in April 1952, with von Kármán as chairman. At the age of 70, he had achieved his postwar ambition — to move back to Europe and live in Paris. AGARD's substantial contributions to aeronautical science lay ahead.

Von Kármán packed a prodigious and varied amount of activity into his life, and Gorn's account concentrates on the main threads. Well researched and prodigally referenced, the narrative holds the reader's attention throughout, with only a few discontinuities to disturb the flow. No new insight could be expected, but the book's main value is in providing an accessible version of von Kármán's complete story.

From Gorn's description of the various stages of von Kármán's life — whether in Budapest, Göttingen, Aachen, Pasadena, Washington, or on the international scene — aspects of his subject's personality emerge: an original thinker, with an ability to communicate; an ambitious scientist, with a touch of arrogance; an unconventional, inspired and inspiring teacher; and an exuberant man, who used his charm to good effect. He threw great parties and enjoyed wine, women and (possibly) song.

Gorn describes a von Kármán persona that will be familiar to aeronautical conference-goers of the 1950s and early 1960s: "the bemused old Hungarian professor with the conspicuous hearing aid, the rumpled clothes, the black beret, and the Rabelaisian wit". But few of those who are old enough to remember him as an elder statesman at conferences, or who struggled as students with von Kármán vortex sheets, will be fully aware of his early days in Europe and his middle years in California and the Pentagon — in short, of his fascinating life. Gorn tells it well. □

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What luck!

Brian Pippard

The Physics of Chance: From Blaise Pascal to Niels Bohr. By Charles Ruhla. Oxford University Press: 1992. Pp. 222. £30, \$55 (hbk); £14.95, \$27.95 (pbk).

CHARLES Ruhla has made a brave and largely successful attempt to explain, almost without using mathematics, the various ways in which chance enters into the operations of the physical world. He has a clear and lively style, and the text is excellently translated from the original French. For those who want more details of the arguments there are mathematical appendices, but they can be ignored without losing the thread. Without saying so, Ruhla seems to have two types of reader in mind — the amateur of science and the physics teacher, and both can be encouraged to read this book, except for Chapter 3, to which I shall return.

Even the deterministic world of newtonian physics was not immune to chance. One cannot repeat an experiment exactly, because random disturbances are unavoidable. Most often these lead only to a degree of scatter about the mean behaviour, but sometimes a tiny initial disturbance may be amplified until the final outcome is totally changed. This is the phenomenon of deterministic chaos which has shaken the physicist's confidence in the predictive power of his theories. In contrast to the generation of complexity in apparently simple systems, the operations of chance can also lead to the simplification of impossibly complex problems, as in statistical mechanics. Ruhla does well to take Maxwell's deduction of the distribution of velocities among gas molecules as a characteristic example, but is unfair to earlier workers when he suggests that Maxwell created the kinetic theory of gases. Clausius, in particular, would have been the first to object (he usually was), since it was his statistical analysis of the distribution of free paths that inspired Maxwell's still more penetrating study.

With the discovery of quantum mechanics, chance usurped the very throne from which determinism had reigned, and Einstein's efforts to restore order had in due course the opposite effect. Bell's theorem, recast in a form that could be tested experimentally, shifted the Bohr-Einstein debate from the abstractions of thought experiments to the realities of the laboratory. Ruhla is an avowed disciple of Alain Aspect, whose beautifully conceived experiments on phonon correlations showed there could be no salvation for deterministic physics except by abandoning other classical

concepts equally dear to the reactionary heart. In a dialogue with M. de la Palice (the epitome of naive common sense, and presumably a familiar figure to all French readers) he shows how inescapable are the paradoxes of quantum mechanics. Thus in a short book primarily devoted to physics he manages to keep in touch with deep problems of metaphysics, from Democritus to the present day.

Why, then, being so concerned to explain basic principles, does Ruhla descend in Chapter 3 to a collection of rules-of-thumb for estimating errors and significance in experimental measurements? I can only guess that he is, like all too many of his colleagues, in thrall to a tradition that considers no measurement complete unless its probable error is stated. This would be a harmless misconception if the principles of error estimation were easily understood. In truth they are difficult to grasp, and physics students at an early stage of their education are force-fed with cookbook recipes that their teachers would shrink from expounding logically. Surely the emphasis should be on devising good

experiments and getting the most out of equipment, leaving the subtleties of gaussian theory to a later stage. Of course, many physicists have to take error analysis seriously; but quite as many (including myself) looking back on their research careers could say with Rutherford: "If your experiment needs statistics, you ought to have done a better experiment". I should have found no fault with Ruhla if he had explained the difference between using a known statistical ensemble to predict experimental results (kinetic theory of gases or quantum mechanics) and inferring a statistical ensemble from a limited number of measurements (theory of errors). This would have been in line with the intentions of the rest of his book; but Student's t-test is wholly irrelevant. There are other faults in this chapter, such as historical inaccuracies, and if another edition is called for I hope Ruhla will rewrite it to the high standard of the rest. □

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Patterns and processes

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Classification, Evolution and the Nature of Biology. By Alec L. Panchen. Cambridge University Press: 1992. Pp. 403. £45, \$80 (hbk); £16.95, \$34.95 (pbk).

THE battle over phylogenetic systematics has eased off, although the hydra continues to raise a threatening head — 'transformed cladism'. Some believe that the true darwinian revolution in comparative biology has only just occurred, with systematists now agreeing to view evolution as an axiom from which to deduce the methods and concepts of phylogenetic systematics. This, they think, was Willi Hennig's original goal when he outlined the principles of cladistics. But transformed or 'pattern' cladists continue to pose heretical questions. How do we know that species exist? How do we learn about descent with modification, if this happened in the phylogenetic past? How do we learn about the underlying process of evolution without having first established a pattern of relative relationships among organisms? Alec Panchen presents a critical review of these and related issues. His text is the product of a distinguished career in vertebrate palaeontology and phylogeny reconstruction, and offers an excellent introduction to comparative biology and its historical background. But by attempting to reconcile too many

divergent issues, the book remains elusive, uncommitted and multi-stranded. It is uncompromising on one issue, however, and that is Panchen's answer to the question: "how do we know?"

The book "is meant to be one long logical argument", a pledge of intellectual allegiance to traditional darwinism, which indicates reservations about transformed cladists. Panchen's central thesis is that comparative biology differs from all other natural sciences by the "taxonomic statement", which is not a natural law or a logical construct but a predictive generalization about individuals, that is, about historical entities or taxa. His argument is that any generalization in biology captures some similarity and hence invariance in a continuously evolving and thus continuously changing world. Such similarity cannot be the outcome of a universal law of nature or a logical relation, both of which would be independent of time and space. In the face of continuous change, relative invariance must — potentially at least — constitute a signal for common ancestry, that is, for a unique historical process. Similarity, nevertheless, can be observed — but because there can be no theory-free observation, the theory explaining regularity of character distribution must not be allowed to influence character analysis. Otherwise, the result will be meaningless.

This is why Panchen agrees with transformed cladists that the reconstruction of patterns of similarity has logical precedence over their explanation by evolutionary theory. Observed similarity among organisms is what needs to be explained (the 'explanandum'); evolutionary theory (the 'explanans') explains relations of similarity by common descent with modification. Indeed, in 1859, Darwin noted that, according to his theory, "unity of type is explained by unity of descent". At the same time, however, Panchen finds transformed cladists guilty of the *a priori* rejection of phylogeny. But this is not true. Instead, with his defence of a 'feedback loop' allowing evolutionary explanations to influence character analysis, Panchen confounds observation and explanation in a way that violates his own premises.

Panchen insists that the explanandum (observed pattern) does not constitute 'evidence' for the explanatory theory (evolution). Instead, he cites vestigial organs as evidence for evolution. But how do we know that snakes lost limbs? Ontogeny may be the answer — reference may be made to posterior limb buds in 'primitive snakes'. How can we know that these are vestigial limbs, rather than initial (primitive) stages in the evolution of limbs? This information cannot be gleaned from the study of limb development in snakes, but must derive from congruence of characters other than limbs that diagnose snakes as squamate reptiles.

For the fossil record, Panchen refers to Reichert's (1837) theory, later confirmed by the study of synapsids, that the primary jaw articulation was transformed to a sound-transmitting apparatus in ancestral mammals. Reichert never made such a proposition. He identified the presence of two visceral arches as the more generalized (gnathostome) condition of form, which differentiates divergently in 'reptiles' and birds (quadrate and articular) as opposed to mammals (incus and malleus). The transformation of the hyomandibula into the stapes is not observed; it was hypothesized (by Gegenbaur in 1870) once evolutionary theory was accepted, and following the insight that mammals constitute a subgroup of gnathostomes.

And how could we contrast patterns of plant or animal distribution caused by partitioning of their original habitat (by continental drift, marine transgressions or mountain building activity) with those caused by dispersal and colonization, without having a hypothesis of organismic interrelationships first? Pattern reconstruction retains logical priority over process explanations, and the best evidence for evolution remains a highly corroborated pattern of order in nature which itself is based on the empirical

analysis of character distribution.

Panchen's book should be read by everybody who seeks an up-to-date introduction to the theory and practice of comparative biology and its significance for evolutionary theory. It shows that the science of comparative biology is alive and well. □

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Last oases?

Peter D. Moore

The Vegetation of Egypt. By M. A. Zahran and A. J. Willis. *Chapman and Hall*: 1992. Pp. 424. £19.95, \$47.50 (pbk).

THE renaissance of interest in global biodiversity makes a number of demands on biologists. They are required to produce much more exhaustive studies of plant and animal taxonomy, especially in the rich but understudied tropics; they must document the geographical ranges of organisms to identify species under particular threat of extinction; they need to know more of the communities in which species are assembled so that they can adopt a habitat approach to conservation; and they have to provide fuller information on ecosystem function in order to develop the necessary techniques for the continued conservation and management of biodiversity.

The plant life of Egypt has been well served in several of these respects. A reasonably good flora exists in the form of Vivi Täckholm's *Students' Flora of Egypt* (University of Cairo, 1972), although this is now in urgent need of revision, and a very valuable documentation of threatened plant species has recently been published by M. N. El Hadidi, M. M. Abd El Ghani and A. G. Fahmy (*The Plant Red Data Book of Egypt. Volume 1. Woody Perennials*, Cairo University Herbarium, 1992). Now, Zahran and Willis have supplied the long needed descriptive account of the vegetation of Egypt.

Lying at the junction of three major floristic provinces (the Saharo-Arabian, Irano-Turanian and the Mediterranean), Egypt has a particularly interesting mix of plant life, and the communities in which species are assembled are therefore of great interest to plant geographers and phytosociologists. Zahran

and Willis have adopted a very broad, non-quantitative approach to vegetation description, reminiscent of that of Sir Arthur Tansley, and have divided the country into geographical regions for the purpose of description rather than classifying the vegetation into types. Thus the book begins with the Mediterranean coastal belt, proceeds southwards through the Western Desert, back north through the Eastern Desert and Sinai, and ends in the Nile region. Within each of these areas the reader is provided with relevant information on geology, physiography, climate and land-use history before embarking on the vegetation itself, and this greatly enhances the value of the work for general travellers interested in botany and ecology.

The vegetation descriptions themselves inevitably involve extensive lists, but the reading is never dull because the authors constantly remind one of their ecological interests and enthusiasm, linking groups of species with underlying ecological factors, such as water supply, salinity gradients or grazing intensity. They have clearly done extensive field work and are acutely familiar with what

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Splendid Isolation: a mangrove in Sinal, Egypt.

seems like every corner of this large country. In the descriptions of some wadis, for example, even the individuals of certain notable trees and shrubs are documented. It is this kind of detail that makes the book valuable as a basis for the further development of national conservation policy.

Egypt has an exceptionally long history of human exploitation of vegetation and the authors have avoided the temptation to idealize their descriptions or to predict 'natural' vegetation. Who can say what the vegetation of the Nile Valley would have been like if water hyacinth had not been introduced and if papyrus had not been exterminated? The stylized graphics of the tombs provide some idea, but Zahran and Willis wisely steer clear of such speculative reconstruction and stick to a thorough description of what

exists without comment on what might have been. Nevertheless, the historical view of vegetation is covered in a chapter by Sekina Ayyad, whose account of Egyptian palaeobotany and palynology underlines the immense opportunities that exist for further work in these two fields.

Any European botanist who has recently visited or worked in Egypt will have been impressed by the intensity of human impact on both the wetland and the arid vegetation. This book provides the basis for further studies on the processes involved in vegetation change and response to these human pressures and it also draws attention to the precarious position of certain vegetation types, such as those found in the saline depression of Wadi El Natrun — possibly the last site

for *Cyperus papyrus* in the whole of Egypt. The site is heavily grazed and is subject to fluctuations in water level and salinity, and the effect of these variables on the survival of this and other scarce species, such as *Typha elephantina*, urgently needs to be studied.

Congratulations are due to Zahran and Willis on the production of a fine book. Let us trust that it results in the fourth and vital stage in biodiversity studies — the development of management techniques for the maintenance of the remaining fragments of this part of the world's plant resources. □

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academy is thus an inevitability. We have no option but to live with positivist geography, Marxist geography, humanistic geography, Islamic geography, structuralist geography, Christian geography, a people's geography and on and on. Each will be within their [sic] cognitive rights to hold to theories that comport with their system of control beliefs." [All are] "as legitimate now as they were in the fifteenth and sixteenth centuries, where my story began."

As Pilate said: "What is Truth?" Are any and all controlling beliefs really equal? Even if a logical demonstration of how words and things match up is impossible, is there no evolutionary strain towards accuracy and adequacy of conceptualization? Don't some ideas work better than others? Are there no restraints on the unbridled imagination?

In fact, Livingstone clearly does believe that the globe is spherical; that Columbus possessed "detailed knowledge of the wind circulation systems of the sea" and that maps have some sort of relation to what exists, since "the emerging map of the world must rank among the finest intellectual achievements of the age of discovery". His insistence that geographers of the fifteenth and sixteenth century were also affected by theology, magic and other contemporary ideas is also familiar. But when he turns to post-Enlightenment geography his deconstruction of old pieties becomes obsessive. He discerns motives (almost always unworthy) for new schools of thought, so that the history of geography becomes a tale of struggles for power among individuals, nations and academic disciplines. That such motives operated seems clear enough; and his account makes older visions of an ever-advancing science of geography look naive. But by dismissing the search for truth, disdaining the accumulation of information, and affirming that disgraceful motives were usually decisive, he looks in his own distorting mirror at the subtle, complex and confused Anglo-American geographical tradition. □

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Obsessive deconstruction

William H. McNeill

The Geographical Tradition. By David N. Livingstone. Blackwell: 1992. Pp. 434. £45, \$55 (hbk); £13.95, \$19.95 (pbk).

DAVID Livingstone's book is a fine example of intellectual history with a vengeance. His subject is geography as practised in the English-speaking world since about 1500, with some side glances at Iberian forerunners and at German and French writers who impinged on the British and American geographical tradition. He brings substantial erudition and intensely self-conscious epistemological sophistication to bear on deconstructing the texts he examines. Yet, since the word 'truth' is absent from his vocabulary, what principally exercised the minds of the writers he discusses — the accuracy and adequacy of their ideas and data in relation to 'reality' — is absent from his account of their doings. Instead he looks for connections between their ideas and other facets of their lives. In his own words: "The heart of my argument is simply that geography changes as society changes, and that the best way to understand the tradition to which geographers belong is to get a handle on the different social and intellectual environments within which geography has been practiced."

Sometimes Livingstone's externalist approach successfully highlights unsuspected dimensions of dead geographers' mentalities. Thus he makes a good case for saying that Carl Sauer's regionalism was "as much as anything else, an exercise in moral recovery". Similarly, he asserts that pre-darwinian geography was divided between "outdoor practitioners and armchair philosophers. As to rhetoric, the former concentrated

their endeavours on the inductive gathering of global data, though this was frequently an exercise in the fabrication of those facts best suited to the national interests of colonial power. By contrast, the theoreticians wanted to infuse such items of geographical particularity with a grander cosmic purpose and to identify in the agents of geographical change the hidden hand of divinity." These and many other judgements strike me as illuminating and convincing.

But all too often, Livingstone prefers denigration. For instance, he declares that the Eurocentric conceptualization of Captain Cook's reports from the south Pacific "became the tools of cultural subjection and the plundering of identity". What plundering an identity means is unclear to me, and I doubt whether Cook's conceptualizations of Polynesian behaviour had such a capability. Or to take a second example: Livingstone accuses Isaiah Bowman of "laying his geographical skills at the feet of the American Council on Foreign Relations and the State Department. Indeed, he himself emerged as an apologist of the American war effort in the 1940s." What does Livingstone think a university president ought to have done when Hitler and Tojo were on the offensive? Does a (very modest) political role vitiate Bowman's "uneasy claims to scientific objectivity" as Livingstone asserts? Or were his shortcomings rooted in epistemological naivety about scientific truth?

Livingstone's own epistemological sophistication does not present a very cheerful prospect. He affirms that "geographers will have to acknowledge that warranted knowledge is relative to a body of beliefs, not to a body of certitudes. Pluralism in the geographical

■ Also just published is *The Challenge for Geography* edited by R. J. Johnston. Chapters cover the global economy (P. Dicken), social landscapes (S. J. Smith), geopolitics (G. Smith), human societies and environmental change (I. Simmons), land transformation (A. Goudie) and climate change (M. Parry). P. Taylor, P. Jackson and R. Abler examine the implications of these changes for the discipline of geography. Blackwell, £50 (hbk), £14.99 (pbk).

Crystal structure of globular domain of histone H5 and its implications for nucleosome binding

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The structure of GH5, the globular domain of the linker histone H5, has been solved to 2.5 Å resolution by multiwavelength anomalous diffraction on crystals of the selenomethionyl protein. The structure shows a striking similarity to the DNA-binding domain of the catabolite gene activator protein CAP, thereby providing a possible model for the binding of GH5 to DNA.

THE linker histones H1 and H5 are components of chromatin which are essential for the organization of nucleosomes into a higher order structure, the 30 nm filament¹⁻³. In addition, evidence is emerging regarding the role of these histones as repressors of transcription⁴. H1 and H5 are homologous^{5,6}, and H5, which is the predominant form of linker histone in avian erythrocytes, may be regarded as an extreme variant of H1. H5 has several lysine-to-arginine substitutions relative to H1, and binds more tightly to chromatin than does H1. Its synthesis in the cell coincides with the maturation of the erythrocyte⁷. Induction of the H5 gene in transfected rat sarcoma cells results in the inhibition of DNA replication and the arrest of cells in G1 phase⁸. In many respects H5 is more similar to histone H1⁹, which is found in terminally differentiated cells⁹, than it is to H1. All these data suggest that H5 is associated with chromatin that is inactive in transcription and replication.

Both H1 and H5 consist of a central, trypsin-resistant, globular domain which is flanked by extended, basic amino- and carboxy-terminal arms^{10,11}. The very basic C-terminal arm comprises almost half the protein, with half the residues in this arm consisting of lysines or arginines. These basic arms are likely to be involved in interactions with linker DNA. The highly conserved globular domain is essential for the binding of H1 to the nucleosome. It confers on the nucleosome the same protection from micrococcal nuclease digestion as full-length H1 (ref. 12), suggesting that the globular domain binds to the nucleosome as an independent module. In H5 bound to chromatin, many lysines in the globular domain are strongly protected against chemical modification whereas the lysines in the N-terminal and C-terminal tails are only weakly protected¹³. This implies that the globular domain has a different and possibly more specific mode of interaction with DNA from the rest of the protein. Although it is generally accepted that the globular domain of H1 or H5 binds to the exit and entry points of nucleosomal DNA, there are currently many conflicting models for the detailed picture of how this is achieved¹⁴⁻¹⁷. In order to understand its binding to the nucleosome, we crystallized GH5, the globular domain of H5 (ref. 18), and report here its structure and implications for nucleosome binding.

Structure determination

The structure was determined by multiwavelength anomalous diffraction (MAD)¹⁹ on crystals of the seleno-methionyl form of the protein. Details of the data collection are shown in Table 1a. Two phasing methods were used to obtain electron density maps. The first used the algebraic formalism developed by Hendrickson and coworkers²⁰; the other treats multiwavelength data as arising from a conventional multiple isomorphous replacement experiment²¹, with the inclusion of anomalous scattering²²⁻²⁴, and uses a maximum-likelihood algorithm^{25,26} to refine heavy-atom parameters. Details of the structure determination are shown in Table 1b and Fig. 1. In our experience,

treatment of the data as a conventional heavy-atom derivative problem, combined with the use of a maximum-likelihood algorithm to estimate heavy-atom parameters, gave somewhat better maps than the algebraic formalism.

Description of the structure

The part of the H5 sequence that was expressed in *Escherichia coli* consists of Met-Thr-Glu (corresponding to the first three codons of H5), followed by residues 22-105 of H5, followed by Leu-Val introduced in the process of inserting a stop codon. The whole polypeptide chain is 89 residues long, and encompasses the globular domain generated by tryptic digestion of H5, which spans residues 22-102 (ref. 27). In the following, all numbering refers to the native H5 sequence.

The asymmetric unit in the crystal consists of two molecules, A and B, shown in Fig. 2a. The two molecules are related by a rotation angle χ of 165.6 degrees and a translation T_x of 2.8 Å. We have built residues 24-96 into the electron density, although poorly defined density is visible beyond residue 96 of molecule A. Thus of the 89 residues in the polypeptide chain, 5 residues at the N terminus and 11 residues at the C terminus are missing in the current model. Each molecule consists of a three-helix bundle (helices I-III), with a β -hairpin at the C terminus. There is also a short three-residue stretch comprising residues 44-46 between helices I and II that is in the β -strand conformation. Together with the C-terminal β -hairpin, this strand forms the third strand of an antiparallel β -sheet. Residues 40-43 are disordered in each monomer.

Figure 2b shows a superposition of the A and B monomers. In the three-helical bundle, the r.m.s. deviation in the positions of the $C\alpha$ atoms is only 0.35 Å, showing that the two molecules are nearly identical. However, the β -hairpin is in a completely different conformation in the two monomers. In the A monomer, the β -hairpin is extended, whereas in the B monomer, it bends in towards the body of the molecule. The reason for this is that in the A monomer, the turn of the β -hairpin is stabilized by intermolecular crystal contacts, mainly through hydrophobic interactions involving Val 87 and Ala 89. This also results in better defined density for the β -hairpin of the A monomer.

The interface between the A and B monomers consists of helices I and II. In particular, there are solvent-mediated interactions involving Tyr 53, Tyr 58 and His 57 from both molecules; these six residues form a cluster at the interface. It is noteworthy, therefore, that Tyr 53 and His 57 are both absent in chicken H1. This may explain the tendency of GH5 but not GH1 to dimerize in solution²⁷. The area of the interface is about 650 Å², which is at the lower end of the range observed for molecules that exist as dimers²⁸. It remains to be seen whether the interaction between the molecules in the crystallographic dimer is representative of the GH5-GH5 interactions in chromatin.

The structure makes clear why certain residues are highly conserved. Glycine at position 79, which is present in all H1

TABLE 1 Structure determination of GH5

(a)									
Wavelength (Å)	f' (electrons)	f'' (electrons)	High-gain data			Low-gain data			N_{unique} (combined)
			N_{measured}	N_{unique}	R_{merge} (%)	N_{measured}	N_{unique}	R_{merge} (%)	
0.9802	-9.52	3.15	13,399	4,450	5.66	10,966	4,533	5.95	6,754
0.9795	-7.35	5.92	14,113	4,516	7.33	10,854	4,508	7.97	6,742
0.9150	-1.89	3.37	13,512	4,543	5.91	11,565	4,602	5.88	6,765

(b)				
Phasing method	Number of reflections phased	Mean figure of merit of phased reflections	Mean figure of merit of all reflections	Map correlation with current model
MADSLQ. Initial:	4,919	0.86	0.63	0.43
after solvent flattening:	6,742	0.85	0.85	0.56
MLPHARE. Initial:	6,635	0.65	0.64	0.54
after solvent flattening:	6,742	0.84	0.84	0.67

a, Data collection: The gene for GH5 was cloned into a plasmid that is part of the T7 expression system³⁸, and we expressed the gene in the *E. coli* strain B834(DE3), which is a methionine-requiring auxotroph with an inducible gene for T7 RNA polymerase on the host chromosome. Fully substituted selenomethionyl protein was produced by growth and induction of these cells in a synthetic medium containing selenomethionine together with the other 19 amino-acids. Crystals of the selenomethionyl protein were isomorphous with those from the native protein obtained previously¹⁸. Data were collected on crystals of selenomethionyl GH5 by rotations about the a^* and b^* axes, using an Enraf-Nonius FAST area detector and the computer program MADNES³⁹ on beam-line X12C at the NSLS at Brookhaven National Laboratory. The crystals were accurately aligned to allow simultaneous measurement of both reflections in a Bijvoet pair. The same reflections were measured on each crystal at three wavelengths, λ_{1-3} , chosen as previously recommended²⁰ to maximize the dispersive and anomalous differences from the selenium K-edge. The position of the absorption edge of selenium was determined before each set of three-wavelength data by the measurement of X-ray fluorescence from the crystal at right angles to and in the plane of polarization of the beam. These data were used to calculate the values of the f'_i and f''_i , the real and imaginary components, respectively, of the anomalous scattering factor of selenium at wavelength λ_i ²⁰. Each three-wavelength batch of data consisted of a rotation range of 8–10 degrees; batches were overlapped by 1 degree. Over 30 crystals were used. To obtain the most accurate data possible, we measured data at two gain settings of the FAST detector: the MADNES parameters to control voltage settings on the camera and image intensifier were SETDET 7.1 at high gain and 3.1 at low gain. At high gain, about 40% of the reflections were flagged as saturated and were discarded, while at low gain, weak reflections (<3 standard deviations) were discarded as unreliable. Data for all three wavelengths were scaled together using the program FBSCALE⁴⁰; however, the high and low gain data were scaled separately. Scale factors were determined to bring these to a common and absolute scale, and to generate a merged set of amplitudes, but the high and low gain data were phased separately. The observed diffraction ratio²⁰ for the Bijvoet differences at λ_2 (which is where the value of f'' is maximal) was 0.111 for acentric reflections, as compared to the expected value of 0.049 at zero scattering angle. However, the diffraction ratio for centric reflections, which should be zero theoretically, was 0.059. Thus over half the magnitude of the Bijvoet differences came from noise in the data. **b, Phasing:** The data were phased by two different procedures. (1) The first uses the algebraic formalism developed by Hendrickson and coworkers¹⁹. In this method, the phase difference $\Delta\phi = \phi_N - \phi_A$ between the phases from the normal and anomalous scattering in the structure is estimated by measuring Bijvoet pairs for a reflection at two or more wavelengths. The positions and occupancies of the anomalous scatterers are determined separately to yield the phase ϕ_A from the anomalous scattering. This phase is used with the phase difference $\Delta\phi$ to estimate the phase ϕ_N from the normal scattering, which is then used to calculate a map of the structure. The phase difference $\Delta\phi$ was estimated using the program MADLSQ²⁰. Cutoffs of $F_A < 150$ and $Q < 7.5$ were used in the phasing of scaled but unmerged data. The values of f' and f'' were refined in the program and did not change significantly from the starting values. The mean value of the residual Q was 3.0; the standard error in the phase difference between the anomalous and normal part of the structure, $\sigma(\Delta\phi)$, was 21°. The sites for two selenium atoms, identified from the anomalous Patterson of the λ_2 data, were refined against Bijvoet differences using the program ANOLSQ²⁰, to give an R of 0.36. Refinement against the F_A values using the program ASLSQ was unstable and not used. The refined heavy-atom parameters were used with the program MADABCD⁴¹ to generate phases and Hendrickson-Lattman phase probability coefficients. Phases of multiply measured reflections were combined using the phase probability coefficients. The mean phase difference between multiple measurements was 38°. About 70% of the reflections were phased by this method; altering the various cutoff criteria in the programs did not noticeably improve the map. Solvent flattening was used to improve the initial map using the package PHASES⁴². (2) We also treated the phasing as a conventional heavy-atom derivative problem²¹ with the inclusion of anomalous scattering^{22,23,24}. Diffraction at one of the wavelengths, λ_1 , is treated as a 'native' data set that has intrinsic anomalous scattering. Data at the other two wavelengths are treated as 'derivatives'. The dispersive term $f'_i - f'_1$ is the source of isomorphous differences, and the anomalous term f''_i , gives rise to Bijvoet differences. Thus the real and imaginary parts of the atomic scattering factors for the 'derivative' at λ_i were set to $f'_i - f'_1$ and f''_i , respectively. For each 'derivative', the real and anomalous occupancies of the selenium atoms were refined separately, using the program MLPHARE²⁵ in its CCP4 implementation⁴³. This program uses a maximum-likelihood method to estimate heavy-atom parameters. As in the first method, the phases for the high and low gain data were combined and used with the $\lambda_1 F_{\text{obs}}$ to calculate a map, and solvent flattening was used to improve the map. To judge objectively the quality of the various maps, we calculated correlations between the various maps and a map generated from the current model. Details of the structure determination will be presented elsewhere.

molecules, is involved in making a sharp bend in the polypeptide chain, in going from the end of helix III into the β -hairpin. The highly conserved residues Leu 81, Phe 93 and Leu 95 form a hydrophobic cluster which anchors the base of the β -hairpin to the core of the three-helix bundle. A number of highly conserved glycines and other small residues at the C terminus of GH5 lie at or close to the turn of the β -hairpin, and presumably are required to form the hairpin without steric hindrance.

Comparison with NMR structure

The overall fold of GH5 has been determined using two-dimensional NMR¹⁴. The topology of the three helices in the NMR structure, and the assignment of the residues that make up the helices agree well with the crystal structure. But the two structures differ completely in detail. The $C\alpha$ r.m.s. deviation between the crystal structure and the NMR structure is 6 Å

overall, and 5 Å when only the three helices are considered for the comparison. The helical axes have significantly different directions in the two structures, and it is not possible to superimpose the helices. The difference between the crystal and NMR structures is not surprising as GH5 was a very early NMR structure, and corresponds to a 'first generation' rather than a 'fourth generation' structure²⁹. In a protein with a fairly high helix content, there were relatively few nuclear Overhauser effect constraints, and the $C\alpha$ r.m.s. deviation between various NMR solutions was 4.3 Å (ref. 14). Thus the NMR structure must be considered as a description of the fold rather than a high-resolution structure.

There are also real differences between the NMR and crystal structures: the NMR structure shows no evidence of any β -sheet. This may be the result of different sample conditions. The NMR analysis was done at pH 3.7 on GH5 that was made by proteo-

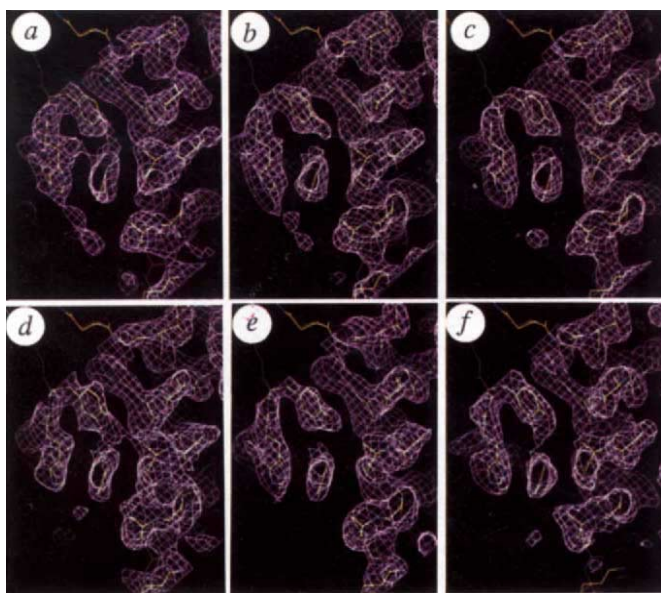


FIG. 1 Electron density maps of a region of GH5. Phases calculated as described (Table 1) were used with amplitudes from the λ_1 data to generate electron density maps using the CCP4 suite of programs⁴³. The region shown includes the helix that contains met-31, which was replaced by seleno-methionine (see text and legend to Table 1) to obtain phases by anomalous dispersion. The refined position of the selenium atom is shown by a green cross. *a*, Map calculated from phases obtained using the algebraic formalism and the program MADLSQ (see text and Table 1); *b*, same map as in *a* after solvent flattening was used to improve the phases; *c*, same map as in *a* after 4 cycles of non-crystallographic symmetry averaging; *d*, map calculated from phases obtained using the conventional multiple isomorphous replacement approach, and the maximum likelihood algorithm for refinement of heavy atom parameters using the program MLPHARE (see text and Table 1). *e*, Same map as in *d* after solvent flattening; *f*, $2F_o - F_c$ map calculated from current model. All maps show electron density contoured at 1 standard deviation, with the current model displayed for comparison. The pictures were generated by the computer program O⁴⁴.

lysis of native H5. The GH5 used in crystallization was obtained by expression in *E. coli*. It was purified in pH 7–8 and crystallized in phosphate buffer at pH 8.0. So it is possible that the β -sheet that is present near physiological pH is disrupted at low pH.

Comparison with CAP

A possible similarity of GH5 with the DNA-binding domain of the catabolite gene activator protein (CAP) has been suggested¹⁴. The DNA-binding domain of CAP consists of residues 135–205 and includes helices D, E and F. But, perhaps because of the irregularity in the second helix of the NMR structure of GH5, it was concluded that there was no correspondence between helix II of GH5 and helix E of CAP and that there was no helix–turn–helix motif in GH5. In fact, as the present work shows, the three helices in CAP and the GH5 crystal structure can be superimposed extremely well (Fig. 3a).

If only those residues that are part of helices are considered, the $C\alpha$ r.m.s. deviation between CAP and GH5 is 1.3 Å. If one considers all structurally equivalent residues, the $C\alpha$ r.m.s. deviation is 1.7 Å, which is well within the range observed for homologous proteins that have only 10% sequence identity³⁰. This result is reinforced by an overall similarity of the topology, even apart from the three-helical bundle. As seen in Fig. 3b, both proteins have a β -hairpin at the C terminus. In CAP there is a second β -hairpin between helices D and E, which forms a 4-stranded antiparallel β -sheet with the C-terminal hairpin. In GH5 there appears to be a deletion, so that the hairpin between helices I and II is replaced by a single short stretch of β -strand; this results in a β -sheet with three, rather than four, strands.

The secondary structure assignments for GH5, and the alignment of the structurally equivalent residues in CAP, are shown in Fig. 4. This alignment differs from those proposed previously. In the alignment based on the NMR model, the alignment for helix III is the same as the one shown here¹⁴; however, the alignment for helix I is shifted by one residue whereas the alignment for helix II, for which no structural similarity was observed in the NMR study, is completely different. Another

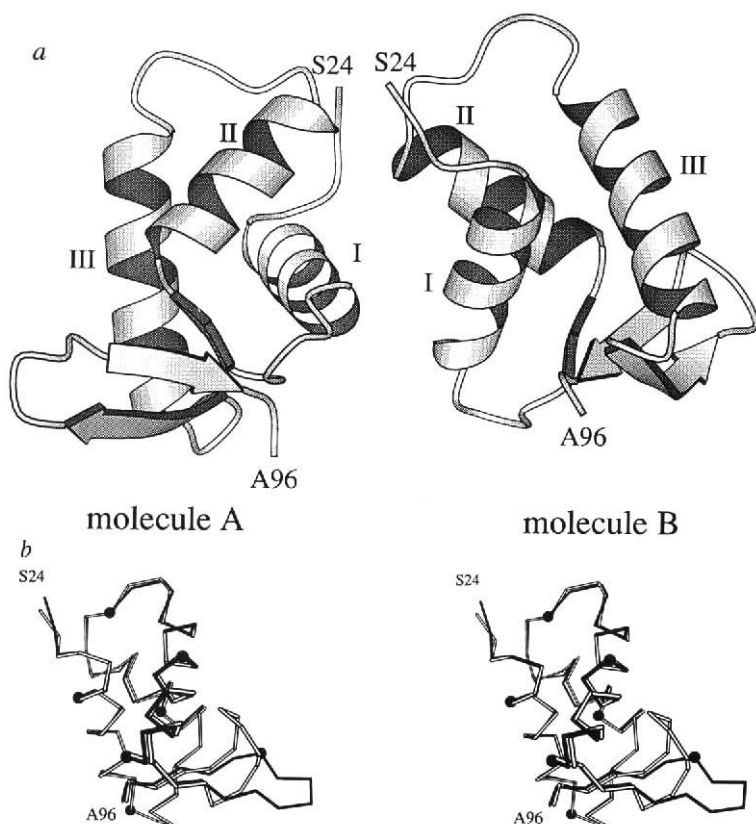


FIG. 2 Structure of GH5. The solvent flattened electron density map (Fig. 1b and 1e) was skeletonized using the program BONES, and the skeletonized map was used with the density to trace the main chain of both monomers in the asymmetric unit using the program O⁴⁴. A monomer mask was generated at this point, and non-crystallographic symmetry averaging using the programs A and O⁴⁴ was used to improve the side chain density in parts of the molecule. The side chains were then built into the molecule. Residues 24–96 were built into the map using the program O. The initial model was refined by simulated annealing using the program XPLOR⁴⁵, followed by restrained *B*-factor refinement. Non-crystallographic symmetry restraints were not applied. The current *R*-factor is 25% using all measured data between 6 and 2.5 Å. But 16 of the 89 residues in the polypeptide chain are missing from the model, mostly at the C terminus, and poorly defined density is visible in the region containing the missing residues. The current model consists of 1,110 non-hydrogen atoms, 18 water molecules and 4 phosphate ions. The deviation from ideality is 0.015 Å in bond length and 3.1 degrees in bond angles. The model is currently being refined against a native data set to 2.3 Å, which should eventually allow more residues to be included in the model. *a*, Schematic diagram of the asymmetric unit of GH5, showing the A (left) and B (right) monomers. *b*, Stereo pair showing the $C\alpha$ trace of the A (solid line) and B (open line) monomers in the asymmetric unit of GH5 superimposed on each other. Every tenth residue beginning with residue 30 is shown by a black sphere. The figures were generated by the computer program MOLSCRIPT⁴⁶.

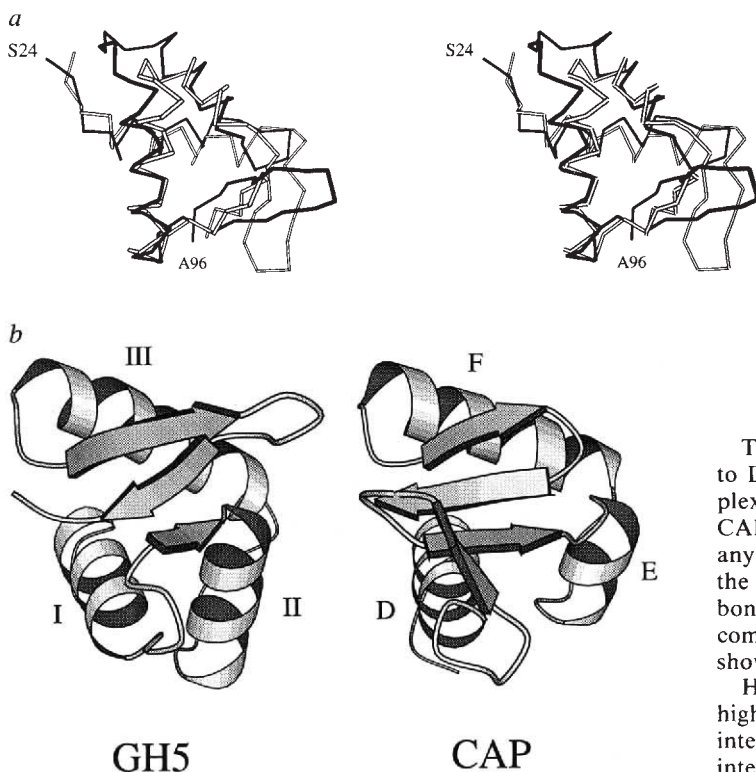


FIG. 3 Comparison of the structures of GH5 and the DNA-binding domain of the catabolite gene activator protein CAP^{31,47}. The superposition was done using the command LSQ_IMPROVE in the program O⁴⁴, which considers pairs of C α atoms within 3.8 Å of each other in the superposition to be structurally equivalent, and does a least-squares minimization of the difference in the positions of structurally equivalent C α atoms to calculate the transformation. *a*, Superposition of the C α trace of GH5 (solid line) and residues 135–205 of CAP (open line). *b*, Schematic ribbon diagrams of GH5 (left) and the DNA-binding domain of CAP (right), showing the similarity in the topology of the two structures. The figures were generated by the computer program MOLSCRIPT⁴⁶.

alignment, based on sequence comparison¹⁷ is also completely different from the one shown here.

DNA binding

The structural similarity between GH5 and the DNA-binding domain of CAP suggests that GH5 and CAP are related proteins. If so, it also suggests that GH5 recognizes DNA in a manner similar to CAP. This is not meant to imply that the details of the interaction are the same in both proteins. The proteins have little sequence identity and obviously diverged a long time ago. CAP is a prokaryotic sequence-specific protein which is known to bind to DNA as a dimer and to induce a bend in DNA. On the other hand, GH5 is a eukaryotic protein that binds as a monomer, is not sequence-specific (although it may have sequence preferences), and the structure of the DNA on binding to GH5 is unknown. Nevertheless, it is likely that the qualitative features of DNA binding are the same. In particular, helix III of GH5, which corresponds to the recognition helix F of CAP, should bind to DNA by fitting into a groove, and the approximate face that GH5 presents to a duplex of DNA should be the same as the corresponding part of CAP.

To understand the qualitative features of the binding of GH5 to DNA, we have taken the structure of the CAP/DNA complex³¹, and made a least-squares superposition of GH5 onto CAP on the basis of the sequence alignment in Fig. 4. To avoid any presumptions about the structure of the DNA, we replaced the bent DNA in the actual complex with the phosphate backbone for B-form DNA that was used to model the CAP/DNA complex³². The resulting model of GH5 binding to DNA is shown in Fig. 5.

How well does this model agree with existing data? First, the highly conserved residues Lys 69 and Arg 73 are positioned to interact with one strand of DNA, and Lys 85 is positioned to interact with the other strand. These residues have counterparts in CAP (Arg 185, Lys 188 and His 199, respectively; see Fig. 4) which interact with phosphates in the corresponding DNA strands. Second, Lys 69 and Lys 85 are protected against chemical modification in chromatin¹³. Lysine 85 is protected almost three times as well as any other lysine in H5. Mutation of this lysine to a glutamine or glutamic acid results in the loss of the globular domain's ability to protect 166-base-pair (bp) nucleosomal DNA from nuclease digestion³³, implying that this residue is involved in DNA binding. Finally, His 25 and His 62 can be crosslinked to DNA in chromatin³⁴. As seen in Fig. 5, these residues are in the vicinity of the same duplex of DNA as the recognition helix, although adjustments to the model will have to be made to bring them into proper position to interact with DNA. This could involve both small changes in the relative orientation of GH5 and DNA, and structural changes in the DNA itself. Thus our model for binding of GH5 to DNA based on the homology with CAP is in agreement with existing biochemical data, but differs from previously proposed models^{15–17}.

Nucleosome binding

In chromatin, GH5 binds to the nucleosome, probably near the dyad axis^{2,12,35}. There is good evidence that *in vitro* GH5 binds

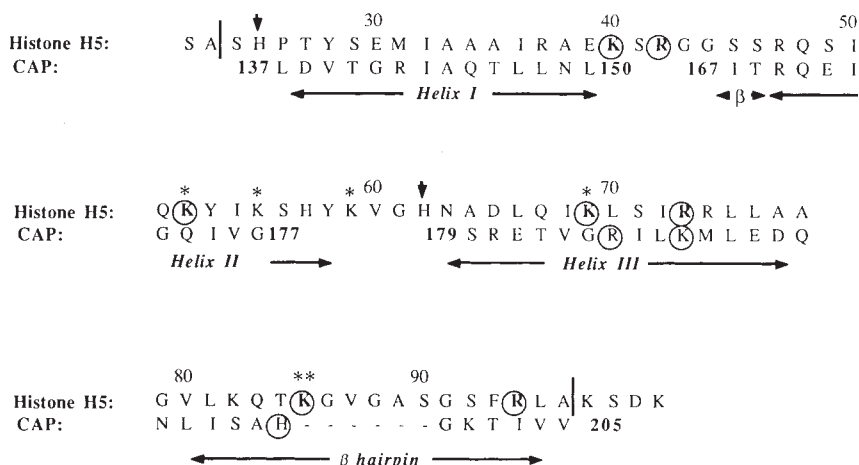


FIG. 4 Secondary structure assignments for the residues of GH5. The vertical bars show the region of H5 that has been built in the electron density map. The alignment with structurally equivalent residues of CAP (defined in legend to Fig. 3) is also shown. In GH5, the circled amino acids in bold face are those that are highly conserved throughout the H1 family. In CAP, the circled amino-acids are known to interact with DNA in the crystal structure of the CAP/DNA complex³¹, and these have counterparts among the conserved basic residues in GH5. Those lysines in GH5 with an asterisk above them are protected against chemical modification in chromatin¹³. Lysine 85, indicated by a double asterisk, was three times as protected as the other lysines. The two histidines (arrowed) have been crosslinked to DNA in chromatin³⁴.

to two duplexes of DNA^{27,36}. The crystal structure shows the presence of two probable DNA-binding sites: in addition to the residues already discussed, there is a second cluster of highly conserved basic residues in GH5, consisting of Lys 40, Arg 42, Lys 52 and Arg 94, which could interact with a second duplex of DNA (Fig. 5). These residues lie ~25–30 Å away from Lys 69 and Arg 73 in the recognition helix. This second group of residues could interact with an adjacent duplex of nucleosomal DNA. The residues Lys 40 and Arg 42 are part of a disordered loop between helices I and II. Therefore we think that GH5 has a primary DNA-binding site, which is similar to that of CAP, and a secondary binding site that is less structurally defined and may consist of a group of basic residues interacting with phosphates on a second DNA duplex. Our model predicts that if the basic residues that form part of the 'secondary' binding site are replaced by neutral residues, GH5 should no longer bind two duplexes of DNA. It may also lose a preference for nucleosomal DNA over naked DNA, although this preference may depend on the structure of the nucleosomal DNA as well as the presence of more than one duplex in the GH5 binding site.

Existing data on the location of GH5 in the nucleosome are conflicting. DNase I protection experiments show a protection of the DNA at the nucleosomal dyad on GH5 binding³⁵, suggesting that GH5 binds directly to the dyad. It is known that the DNA at the dyad has a minor groove at the exposed surface³⁷. In the model shown in Fig. 5, it is possible that the recognition helix fits into a wide minor groove rather than a major groove as shown, but such a binding of a helix-turn-helix motif would be unprecedented. Direct binding of the recognition helix at the dyad also raises the question of how GH5 protects both entry and exit points of nucleosomal DNA with only two DNA-binding sites, one of which binds at the dyad. It is possible that the protection at the dyad is a result of indirect effects such as steric exclusion. Crosslinking experiments³⁴ place His 25 near the end of the nucleosomal DNA and His 62 in linker DNA. If so, this would place the body of the nucleosome just above the molecule of GH5 in Fig. 5. Each DNA-binding site on GH5 could interact with a region of linker DNA near the entry and exit points of nucleosomal DNA. This would explain the protection by GH5 of nucleosomal DNA against micrococcal nuclease digestion; it would also be consistent with the GH5–DNA crosslinks and with the presence of two binding sites. The details of the interaction of GH5 binding to the nucleosome will become clear only with more data.

The crystal structure presented here suggests that GH5 has a

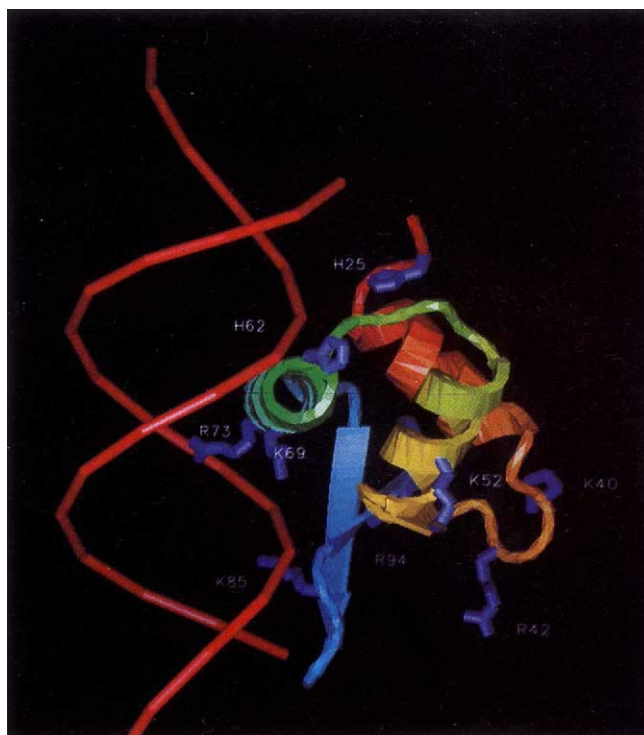


FIG. 5 Model of GH5 binding to DNA, based on the similarity of GH5 with CAP and the known DNA-binding mechanism of CAP. The coordinates of GH5 were superimposed onto those of CAP, and the actual DNA in CAP, which is bent, was replaced by a phosphate backbone trace of B-form DNA (shown in red) to avoid any presumptions about the nature of the DNA. The GH5 molecule is shown colour-ramped from red to blue, going from N terminus to C terminus. Conserved basic residues, as well as the two histidines that have been crosslinked to DNA, are shown in blue. Lysine 85 is closer to the DNA strand complementary to the one that is close to Lys 69 and Arg 73, although this is not obvious in the view shown. The figure was generated by the program O⁴⁴.

primary mode of binding to DNA based on the similarity with CAP, and the presence of a secondary site that could bind to another duplex of DNA. This provides a basis for site-directed mutagenesis and co-crystallization experiments aimed at understanding the binding of GH5 to the nucleosome. □

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Imaging of a Lyman- α absorption cloud in front of the radio galaxy 4C41.17

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THE quasar absorption lines known as the Lyman- α forest reveal the presence of a population of extragalactic objects, thought to be clouds of gas or gas-rich protogalaxies. The gas seems to be highly ionized ($N_{\text{H I}}/N_{\text{H II}} \approx 10^{-4}$), with column densities ($N_{\text{H I}}$) ranging from 10^{12} to 10^{18} cm^{-2} and Doppler parameters of 15–40 km s^{-1} (refs 1–3). Different types of measurement have yielded strongly discrepant numbers for the typical diameter, from several tens of kiloparsecs^{4,5} to only ~ 5 parsecs^{6,7}. Nothing is known about the clouds' shape (spheres or sheets), which could provide another clue to their origin⁸. The observation of huge, luminous Ly- α haloes around some high-redshift radio galaxies^{9–11} provides a new way of obtaining information about Lyman cloud size and shape: the large sizes (many tens of kiloparsecs) and broad emission-line profiles of these haloes make them ideal light screens against which absorbing clouds at similar redshift might be seen as dark shadows. We report here the possible detection of such an absorbing cloud in front of the radio galaxy 4C41.17; in the simplest interpretation, the cloud has an elongated shape, and dimensions of $\sim 40 \times 10$ kiloparsecs.

The giant, high-redshift ($z=3.80$) Ly- α halo object 4C41.17 (ref. 11) extends over an area of about $135 \times 90 \text{ kpc}$ ($1''$ corresponds to 9.1 kpc for $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $q_0 = 0.2$). In order to investigate in detail the morphological and kinematical structure of its extended Ly- α emission, we installed a Fabry-Pérot (FP) etalon in the focal reducer of the 3.5-m telescope on Calar Alto, Spain. We used a camera focal ratio of $f/1.6$, providing a scale of $0.55'' \text{ pix}^{-1}$ on our $15 \mu\text{m}$ charge-coupled device (CCD) chip. The observations were carried out in March 1990 under good weather conditions and at a mean seeing of $1.4''$. We took 13 images with exposure times of 3,000 s within the wavelength range from 580.0 to 587.5 nm, at an instrumental resolution of 1.0 nm: for order separation, a 20.5-nm-wide pre-filter has been used, centred at 584.0 nm. Details of the instrument, observational technique and data reduction are given in ref. 12. In addition we took blue and red frames (FP etalon

removed) with total integration times of 600 and 900 s respectively, and a line-free continuum frame using a 21-nm broad filter centred at 604 nm and exposed for 1,000 s.

The single exposures were overlaid to a common position and to a common effective point spread function (PSF) by convolving with appropriate gaussians and reducing the pixel size to $0.276''$ in x and y . We used a PSF with $1.9''$ full-width half-maximum (FWHM) defined by the frame with the worst seeing. The alignment of all object frames, including the broad band images, could be done within an accuracy of $0.03''$ in both coordinates. By means of the count rates of faint objects in the field, the exposures were scaled to the same count level (accuracy $\sim 1\%$), which is necessary to compare the frames taken at different wavelengths. The scaling was confirmed by spectrophotometry of a $V = 17.6 \text{ mag}$ K1 star $44''$ east of 4C41.17. Absolute intensity calibration was done with standard stars observed with the same set-up as the FP frames.

The R-band image shows a fuzzy object elongated towards position angle (PA) $\sim 80^\circ$, with a size of $1.3'' \times 0.7''$ (after deconvolution of seeing) and a brightness of $22.4 \pm 0.3 \text{ mag}$.

To obtain pure emission line images we subtracted the 604 nm continuum from the FP frames. Adding up all FP frames gives an image shown in Fig. 1a. At our 3σ level of $1.1 \times 10^{-20} \text{ W m}^{-2} \text{ arcsec}^{-1}$ (lowest contour), it smoothly covers an elliptical area of $13'' \times 10''$.

The radio galaxy has the morphology of a FR II source and consists of two lobes and several knots close to the center of the Ly- α halo¹¹, fairly well aligned with the major axis of the optical extended emission line region (EELR). Our position of the peak emission, as derived from measuring 19 positional reference stars¹³ on the Palomar Sky Survey, is $6 \text{ h } 47 \text{ min } 20.799 \text{ s} + 41^\circ 34' 3.83''$, with an uncertainty of $0.25''$. This position is somewhat closer to the adopted centre of the radio galaxy than the position given before¹¹.

The Ly- α emission is brightest on the exposure at 583.7 nm ($z = 3.800$). The images taken at 582.9 nm and 583.0 nm, about 0.7 nm blue-wards of the line maximum of the Ly- α halo, reveal a feature with significantly lower intensity at about $2''$ west from the centre, extending from south to north over a strip of $>3''$ (see Fig. 1b). This is the kind of feature we are looking for, which could be an absorbing Ly- α cloud in front of 4C41.17. To explore this possibility we have to study the line profiles at every position in the halo.

For this purpose all FP images were stacked together to a x - y - λ data cube and a gaussian profile was fitted along the λ axis at every x, y position. The resulting maps of velocity and width

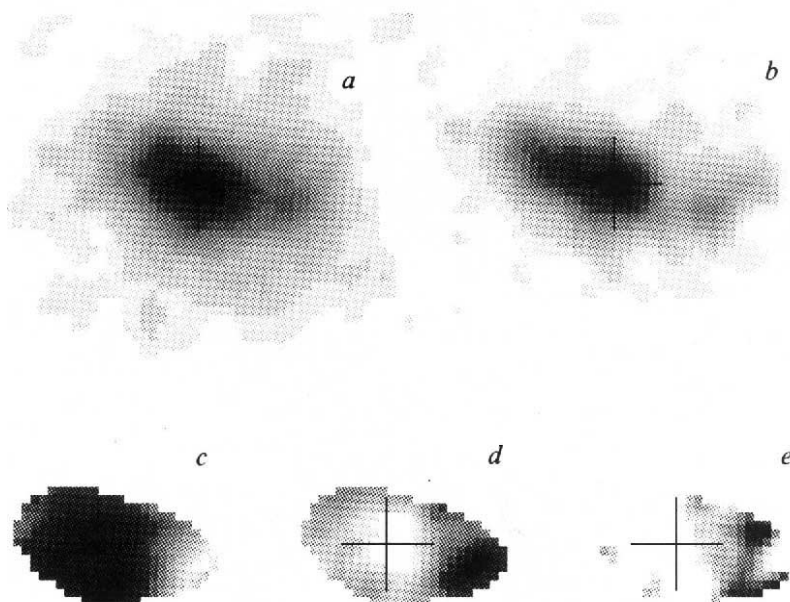
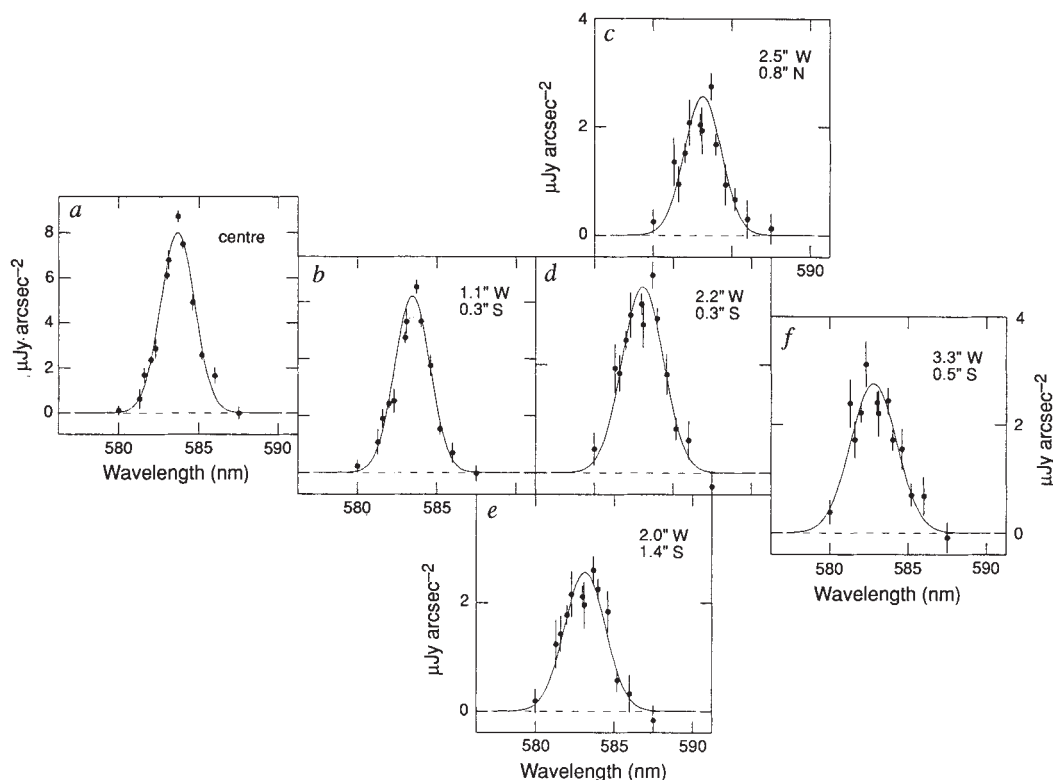


FIG. 1 Ly- α emission halo of 4C41.17. *a*, Sum of all emission line frames. *b*, Sum of frames taken at 582.9 and 583.0 nm. *c*, Radial velocity relative to the centre, ranging from -530 (white) to $+90 \text{ km s}^{-1}$ (black). *d*, Velocity width (FWHM, not corrected for instrumental resolution), ranging from 750 (white) to $1,250 \text{ km s}^{-1}$ (black). *e*, Map of the equivalent width of the Ly- α absorption in 4C41.17 with values ranging from 0.05 to 0.25 nm . The cross that marks the centre of the halo has a size of $3 \times 3''$. North is up.

FIG. 2 Ly- α profiles for the centre (a) of 4C41.17 and for the western part (b-f) averaged over areas of $1.1'' \times 1.1''$. Circles mark the individual measurements and the bars indicate the total error, including all errors from detector readout noise to scaling uncertainties. The solid lines are gaussian fits. The abscissae are scaled in steps of $2 \mu\text{Jy arcsec}^{-2}$ for each profile.



for the central part of the Ly- α halo are shown in Fig. 1c and d.

Figure 2 shows line profiles at several positions in the western half of the emission line cloud: panel a shows the line profile at the brightest region in the halo. The line has an almost gaussian shape with a FWHM = 2.6 nm, which is much more than the instrumental resolution. At $1.1''$ to the west (b) only a marginal change in the profile of the line is observed. Going about $2.2''$ to the west, three plots (c, d, e) give the profiles for a strip from north to south for areas separated by $1.1''$. Here we see a distinct depression at 583.0 nm. The depth of this feature seems to be more or less constant along the strip.

The dip in the line profile could be caused by either (1) the superposition of two emission line clouds with different mean velocity, or (2) an absorbing cloud in front of a region emitting a broad line. These alternatives are compared in Fig. 3. The fit with two gaussian components of a fixed width $\Delta\lambda = 2.6$ nm (the minimum value observed) but free mean wavelengths and intensity yields an unsatisfactory fit to the central 10 data points ($\chi^2 = 6.9$ with five degrees of freedom, corresponding to a confidence level of 22%). Alternative (2) with fixed width of the absorption line of 1.0 nm (instrumental profile width) leads to a significantly lower $\chi^2 = 5.2$ (confidence level 41%). The fact that we see a depression, rather than an enhancement at this position on the images, additionally favours the second alternative.

The general smoothness of the images and line profiles together with the large velocity spread of $1,300 \text{ km s}^{-1}$ (FWHM) indicates that the entire emission line region is made up of numerous sub-clouds, with small intrinsic line widths, moving at large random velocities with respect to each other. The possibility cannot therefore be excluded that a chance deficit of clouds in the velocity bin corresponding to $\lambda = 582.75$ nm causes a dip, which would look like an absorption feature. When we estimate the typical number of sub-clouds on any line of sight from a random velocity distribution, that is, $N_{\text{cloud}} \sim (\sigma/\Delta v)^2$ (where $\Delta v \leq 80 \text{ km s}^{-1}$ is the observed r.m.s. fluctuation between adjacent lines of sight), we get $N_{\text{cloud}} \geq 50$. The probability of finding

a deficit of 30% in a 1-nm broad bin near the peak of the velocity distribution on any line of sight is then about 20%. It is therefore highly improbable ($P \leq 1\%$) that such a deficiency occurs in three neighbouring independent lines of sight.

This implies that absorption by a cloud is the most likely explanation for the observed dip. From the fit (Fig. 3b) we derive an equivalent width of $W = 0.3 \pm 0.1$ nm. In order to map the extent of the absorption feature, we estimate the equivalent width at every position by subtracting the measured brightness at 582.8 nm from the fitted value at this wavelength (omitting the data around 583 nm from the fit). The difference is divided by the fitted brightness and multiplied by $\Delta\lambda = 1.0$ nm (the instrumental resolution) to obtain W .

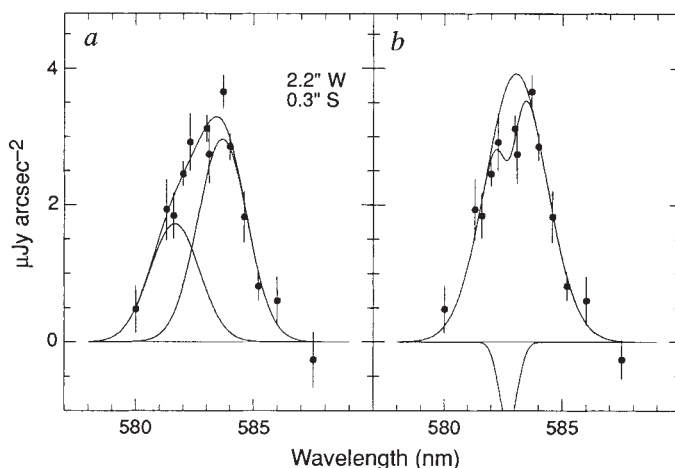


FIG. 3 Fits to the depression in the Ly- α profile. a, The superposition of two emission line components with the narrowest width (FWHM = 2.45 nm) found in 4C41.17, and with a separation of 2.0 nm. b, The superposition of a broad gaussian (FWHM = 3.0 nm), centred at 583.05 nm, with a 0.3-nm blue-shifted narrow absorption line which is broadened to the width of the instrumental profile and has an equivalent width W of 0.3 nm.

The resulting map (Fig. 1e) shows that the absorption feature has a mean W of 0.2 nm and stretches from roughly north to south across the entire emission line region, corresponding to a length of >30 kpc (the map presents only the area with good signal-to-noise ratio). Its spatial width is rather uncertain, because it is unresolved in the east-west direction ($<1.5''$). It cannot be narrower than $0.5''$ because otherwise even a 100% obscuration would be washed out by our beam into a relative depression of $<25\%$ (0.25 nm). So we assume a projected size of 10×10 kpc² for the absorber, with a deconvoluted equivalent width of about 0.5 nm.

Such an absorber can either consist of one or more clouds located well in front of 4C41.17 (if the blueshift of the absorption is cosmological, the absorber sits at a comoving distance of 5 Mpc). On the other hand, the velocity in the EELR itself is large enough to cover this blueshift; otherwise we would be unable to detect the feature. Thus, a dense, partially ionized cloud at the edge of 4C41.17 could equally explain the absorption. In this latter case more detailed observations are necessary for a physical interpretation. We therefore would like to pursue the former possibility of a physically separated absorber. Such clouds—commonly known as Lyman-forest clouds—and their properties have been extensively studied in the absorption line spectra of high redshift quasars.

For comparison we make use of a spectrum¹⁴ of the quasar Q0000-263 ($z=4.11$), the Lyman-forest of which covers the λ range of our observation. We smoothed the original spectrum (resolution, 0.1 nm) to our instrumental resolution of 1.0 nm. The comparison between smoothed and original spectrum reveals that any absorption feature as deep as that observed in 4C41.17 typically consists of two or more narrow absorption lines. We have to realize therefore, that our 'absorption cloud' is likely to be a superposition of several individual Lyman-forest clouds. Nevertheless, we believe that the outline of the absorber in the W map (Fig. 1e) is most likely to be determined by one single cloud which made the feature strong enough to become detectable, and we assign half of the measured equivalent width (0.25 nm) to this cloud. Assuming a Doppler parameter $b=35$ km s⁻¹ and $N_{\text{H I}}/N_{\text{H II}}=10^{-4}$ as typical for Lyman clouds of that depth (refs 1, 3), we find a column density $N_{\text{H I}} \sim 10^{15}$ cm⁻². A cigar-shaped cloud of 40 kpc length and 10 kpc diameter would contain a total hydrogen mass of $\sim 3 \times 10^7 M_{\odot}$.

What is the probability of detecting such an absorption feature in front of 4C41.17? Both the smoothed spectrum of Q0000-263 (ref. 14) and the standard $dN(W, z)/dz$ relation¹⁵ yield ~ 25 features with $W > 0.4$ nm on each line of sight and within one z unit at the observed wavelength. Considering the 'useful' wavelength range of ~ 1.2 nm (the blue half of the width of the emission line) in our search for line features, and the area of the EELR inspected of ~ 20 arcsec², the probability of detecting a cloud of a typical size of a few arcsec² is close to 1.

In conclusion, we believe that we have succeeded in obtaining the first direct observation of a Lyman absorption cloud. Either this cloud belongs directly to the mass concentration around 4C41.17 or it is a physically separated foreground object. In the latter case it would represent the population of Lyman-forest clouds known from the absorption spectra of quasars. In either case, our observations indicate that the relevant absorbers have projected sizes of some 100 kpc² and an elongated shape, like a cigar or a sheet seen almost edge-on in the case of 4C41.17. $\square$

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Superconductivity at 94 K in $\text{HgBa}_2\text{CuO}_{4+\delta}$

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FOLLOWING the discovery¹ of high-transition-temperature (high- T_c) superconductivity in doped La_2CuO_4 , several families of related compounds have been discovered which have layers of CuO_2 as the essential requirement for superconductivity: the highest transition temperatures so far have been found for thallium-bearing compounds². Recently the mercury-bearing compound $\text{HgBa}_2\text{RCu}_2\text{O}_{6+\delta}$ (Hg-1212) was synthesized³ (where R is a rare-earth element), with a structure similar to the thallium-bearing superconductor $\text{TlBa}_2\text{CaCu}_2\text{O}_7$ (Tl-1212), which has one TlO layer and two CuO_2 layers per unit cell, and a T_c of 85 K (ref. 2). But in spite of its resemblance to Tl-1212, Hg-1212 was found not to be superconducting. Here we report the synthesis of the related compound $\text{HgBa}_2\text{CuO}_{4+\delta}$ (Hg-1201), with only one CuO_2 layer per unit cell, and show that it is superconducting below 94 K. Its structure is similar to that of Tl-1201 (which has a T_c of <10 K)⁴, but its transition temperature is considerably higher. The availability of a material with high T_c but only a single metal oxide (HgO) layer may be important for technological applications, as it seems that a smaller spacing between CuO_2 planes leads to better superconducting properties in a magnetic field⁵.

The samples were prepared by solid state reaction between stoichiometric mixtures of $\text{Ba}_2\text{CuO}_{3+\delta}$ and yellow HgO (98% purity, Aldrich). The precursor $\text{Ba}_2\text{CuO}_{3+\delta}$ was obtained by the same type of reaction between BaO_2 (95% purity, Aldrich) and CuO (NormaPur, Prolabo) at 930 °C in oxygen, according to the procedure described by De Leeuw *et al.*⁶. The powders were ground in an agate mortar and placed in silica tubes. All these operations were carried out in a dry box. After evacuation, the tubes were sealed, placed in steel containers, as described in ref. 3, and heated for 5 h to reach ~ 800 °C. The samples were then cooled in the furnace, reaching room temperature after ~ 10 h.

The formation of the new phase $\text{HgBa}_2\text{CuO}_{4+\delta}$ was revealed by X-ray powder analysis, performed with a Guinier-Hägg focusing camera and Fe K α radiation (1.93730 Å). Finely powdered silicon ($a=5.43088$ Å at 25 °C) was used as an internal standard. The intensities of the reflections were evaluated by means of an automatic film scanner and indexed on a tetragonal cell with lattice parameters $a=3.8797$ (5) Å, $c=9.509$ (2) Å and assignment $Z=1$. No systematic absences were observed, leading to the number of molecules per unit cell of the space group $P4/mmm$. The c parameter corresponded to the value calculated from the formula $c \approx 9.5 + 3.2(n-1)$, similar to that deduced for the $\text{TlBa}_2\text{R}_{n-1}\text{Cu}_n\text{O}_{2n+3}$ homologous series. We took this as a strong indication that the powder pattern corresponded to that of the first member of the $\text{HgBa}_2\text{R}_{n-1}\text{Cu}_n\text{O}_{2n+2+\delta}$ series.

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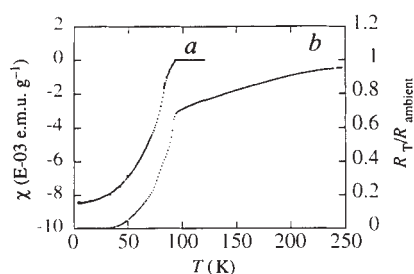


FIG. 1 AC magnetic susceptibility χ (a) and normalized resistivity (b) as a function of temperature for $\text{HgBa}_2\text{CuO}_{4+\delta}$.

Scanning electron microscopy using a JEOL SM 840A equipped with an energy-dispersive spectroscopy (EDS) attachment revealed that the sample was well crystallized with particle sizes of several micrometres. EDS analysis of several well crystallized, flat and oriented grains was performed. Beside Hg, Ba, Cu and O, no other element was detected in the spectra. The average metal ratio found for eight grains was Hg:Ba:Cu = 28(1):47(2):25(1), where the numbers between parentheses are the standard deviations. Determination of the oxygen content by EDS analysis was not possible, so it was estimated by structural analysis and iodometric titration. The cation stoichiometry is in qualitatively good agreement with the proposed formula of the new compound.

Alternating-current magnetic susceptibility measurements between 4.2 and 120 K, done without any additional oxygen treatment, showed that $\text{HgBa}_2\text{CuO}_{4+\delta}$ samples undergo a transition from paramagnetic to diamagnetic with an onset as high as 94 K (Fig. 1a, where the susceptibility is in electromagnetic units g^{-1}). The estimated magnetic susceptibility at 4.2 K. corresponds to >50% of the ideal diamagnetic values.

The resistivity was measured between 4.2 and 250 K by the four-probe technique. The sample was a pressed pellet which was annealed in oxygen for 2 h. The temperature dependence of the normalized resistivity, shown in Fig. 1, exhibits a sharp drop at T_c , but the transition is broad and it reaches the value of zero resistance only at 35 K. This behaviour indicates that the sample is not homogeneous.

To determine the structure of $\text{HgBa}_2\text{CuO}_{4+\delta}$, X-ray powder data were collected by a $\theta/2\theta$ STADI P diffractometer in transmission mode. The experimental conditions were as follows: 2θ range = $6-115^\circ$ (0.02° steps) with fixed counting time 60 s and a rotating sample. An absorption correction was applied and the sample thickness was calculated from the primary beam absorption ($\mu R = 1.7$, where μ is absorption coefficient and R is thickness). The structural refinements were done by the Rietveld method. The initial positional parameters were deduced from a structural model containing the sequence (Hg)(BaO)(CuO₂)(BaO)(Hg). After convergence (intensity discrepancy factor, $R_1 = 0.039$), a Fourier difference map revealed that the position at $(\frac{1}{2}, \frac{1}{2}, 0)$ of the Hg layer was partially occupied. During the final cycle of refinement, the occupancy factor of a third oxygen atom placed in this position was varied together with the positional and thermal parameters for all atoms (except for the thermal parameter of O(3) which was kept fixed at $1.0 \text{ \AA}^2$). The final intensity (R_1) and profile (R_p) discrepancy factors based on 84 reflections were $R_1 = 0.0367$ and $R_p = 0.116$, with a GOF (goodness of fit) = 0.33.

The final positional and thermal parameters together with the relevant interatomic distances are given in Table 1. Observed, calculated and difference diffraction patterns are shown in Fig. 2. A schematic representation of the structure is shown in Fig. 3. Preliminary structural refinements based on powder neutron diffraction data support the presence of oxygen in the O(3) position with an occupancy factor slightly larger than that found by X-ray powder diffraction data. The neutron data also

TABLE 1 Crystallographic data for $\text{HgBa}_2\text{CuO}_{4+\delta}$

Positional, thermal and occupancy parameters

Atom	Position	x	y	z	$B_{\text{iso}} (\text{\AA}^2)$	Occupancy
Hg	1a	0	0	0	2.55 (5)	1.00
Ba	2h	0.5	0.5	0.2979 (1)	1.43 (4)	1.00
Cu	1b	0	0	0.5	0.88 (9)	1.00
O(1)	2e	0.5	0	0.5	0.4 (3)	1.00
O(2)	2g	0	0	0.206 (2)	2.2 (3)	1.00
O(3)	1c	0.5	0.5	0	1.0	0.10 (3)

Selected interatomic distances ($\text{\AA}$)

Hg-O(2) ($\times 2$)	1.95 (2)	Cu-O(1) ($\times 4$)	1.940 (1)	Ba-O(1) ($\times 4$)	2.730 (1)
Hg-O(3)*	2.742 (1)	Cu-O(2) ($\times 2$)	2.79 (2)	Ba-O(2) ($\times 4$)	2.880 (5)
				Ba-O(3)*	2.831 (1)

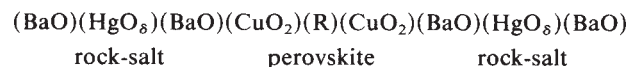
Data obtained using monochromatized $\text{CuK}\alpha_1$ radiation ($\lambda = 1.54056 \text{ \AA}$), giving $a = 3.87766(4) \text{ \AA}$, $c = 9.5073(1) \text{ \AA}$.

* Partially occupied sites.

confirm the large value for the mercury thermal factors. As in the case of the X-ray data, the anisotropic model shows a very slight difference between $B_{11} = B_{22}$ and B_{33} , the thermal factors along x, y and z respectively.

$\text{HgBa}_2\text{CuO}_{4+\delta}$ has a structure related to that of Hg-1212 (ref. 3). Its lattice parameters correspond to four-layered packing along the c-axis of a unit cell: $a = a_{\text{per}}$, $c = 2a_{\text{per}}$ (where a_{per} is the parameter of the perovskite subcell) and its structure contains the sequence (CuO₂)(BaO)(HgO_δ)(BaO)(CuO₂). The Cu cations are octahedrally coordinated, while the coordination of the other cations depends upon the value of δ . This, as obtained from powder X-ray data, is 0.10(3). An important consequence is that most of the Hg cations have two oxygen atoms near them in a 'dumb-bell' configuration, an appropriate coordination for Hg^{2+} cations. Because δ is small and different from zero (within about three standard deviations) X-ray powder data alone are insufficient to determine which sites of the rock-salt positions in the HgO layer are occupied and how they affect the Hg coordination. The extra oxygen atoms are needed in order to increase the average oxidation number of the Cu and to create the concentration of holes necessary for superconductivity. Iodometric titration performed with a large excess of KI leads to 16% of Cu^{3+} , corresponding to $\delta = 0.08$.

Similarly, the structure of $\text{HgBa}_2\text{RCu}_2\text{O}_{6+\delta}$ (the second member of the $\text{HgBa}_2\text{R}_{n-1}\text{Cu}_n\text{O}_{2n+2+\delta}$ series) can be described as six-layered blocks made of rock-salt and perovskite-type structures. In the structure of Hg-1212 the layer sequence is:



The CuO₂ monolayer in Hg-1201 has been replaced by the (CuO₂)(R)(CuO₂) block. As a consequence the Cu cations are

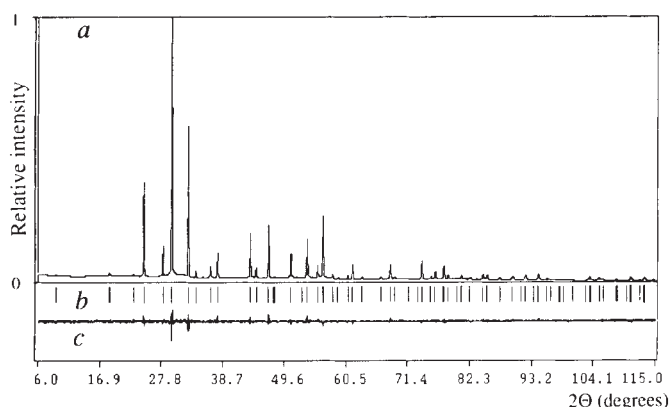


FIG. 2 Observed (a), calculated (b) and difference (c) powder patterns after Rietveld refinement for $\text{HgBa}_2\text{CuO}_{4+\delta}$.

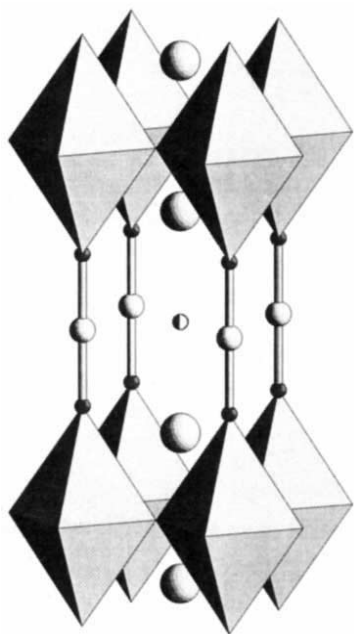


FIG. 3 Structure of $\text{HgBa}_2\text{CuO}_{4+\delta}$. The large, medium and small circles represent the Ba, Hg and O atoms, respectively. The Cu atoms are inside the octahedra. Note that the partially occupied oxygen O(3) site on the Hg layer is represented by a partially filled circle.

pyramidally coordinated. The coordination of the Ba and Hg cations in Hg-1212 is similar to that of the same cations in Hg-1201. The R cations are surrounded by 8 oxygen atoms arranged as a prism. The valence of the Cu cations depends upon the value of δ and the valence of the R cations: if the same Cu valence or hole concentration as in Hg-1201 is needed to induce the superconducting state in Hg-1212, then the R cations should be $2+$ and δ_{1212} should be appreciably greater than δ_{1201} . For the previously reported Hg-1212, R was a mixture of Eu and Ca, and δ was not precisely determined³. It is possible that δ was not large enough to compensate for the higher valence of the R cations and to transfer the needed extra charges to CuO_2 layers.

As stated above, the structural arrangement of $\text{HgBa}_2\text{CuO}_{4+\delta}$ is similar to that of $\text{TlBa}_2\text{CuO}_{5-\delta}$, except for the oxygen stoichiometry of the HgO_8 and $\text{TlO}_{1-\delta}$ layers respectively. For the former, δ is very small and this depletion is possible because the dumb-bell coordination is appropriate for the Hg^{2+} cations. For the latter, the $\text{TlO}_{1-\delta}$ layer is only slightly oxygen depleted, creating the appropriate coordination for the thallium cations, resulting in either a distorted octahedron or a five-coordinated polyhedron. These different requirements for attaining the optimal concentration of holes are due to the different preferred coordination geometries of the Tl^{3+} and Hg^{2+} cations.

The first member of the latter series (Tl-1201) has been reported and found to become superconducting at <10 K (ref. 4). By doping the Ba sites with La this value can be increased to 52 K (ref. 7). The second member of the mono-Tl series becomes superconducting at 85 K (ref. 2). This increase is a general rule for the first few members of this series of compounds. If this behaviour holds for the Hg-series, the second member could reach values for T_c as high as those of the thallium.

The possible advantages for technical applications of $\text{HgBa}_2\text{CuO}_{4+\delta}$, in analogy with one-Tl-layer materials, would be due to the relatively short distance between CuO_2 layers. This might lead to lower anisotropy of the superconducting properties and to higher flux-melting temperatures than those of two-TlO-layer superconductors⁵. □

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Dependence of aggregate morphology on structure of dimeric surfactants

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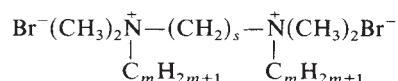
SURFACTANT molecules in water form organized assemblies of various shapes, such as micelles and bilayer lamellae, which are of interest as analogues of biological structures, as model systems for studying complex phase behaviour and because of their technological importance, for example to the food and paint industries. The polar head groups are usually arranged randomly at the surface of these assemblies. We have studied the effect on the microstructure of these assemblies of imposing constraints on the head-group spacing. We investigate the structures formed by 'double-headed' surfactants in which two quaternary ammonium species ($\text{C}_m\text{H}_{2m+1}\text{N}^+(\text{CH}_3)_2$) are linked at the level of the head groups by a hydrocarbon spacer (C_sH_{2s}). Here we report the microstructures formed by these dimeric surfactants with $m=12$ and $s=2, 3$ or 4 in aqueous solution, by rapidly cooling the micellar solutions and investigating the vitrified structures with transmission electron microscopy. The surfactants with a short spacer ($s=2, 3$) form long, thread-like and entangled micelles even at low concentrations, whereas the corresponding monomeric ammonium surfactants can form only spherical micelles. The dimeric surfactants with $s=4$ form spheroidal micelles. Thus short spacers (which impose reduced head-group separation) appear to promote lower spontaneous curvature in the assemblies. This approach may afford a new way to control amphiphile self-aggregation.

Conventional surfactant molecules generally comprise two distinct parts that are incompatible with each other: one polar head and either one or two alkyl chains. These molecules tend to self-associate in water, where they produce micellar solutions in the dilute range, and lyotropic mesophases at higher concentrations. Whatever the structure, the surfactant polar heads are located at the interface between the hydrocarbon and water regions. Their relative positions and distances are determined mainly by their electrostatic interactions, and also by the packing requirements of the disordered alkyl chains^{1–3}. In caesium or rubidium soaps at low temperature in the presence of water, for example the head groups form well developed hexagonal or rectangular crystalline arrays⁴. Generally, however, they are arranged randomly, and little is known of their packing geometry or the width of their spacing distribution.

To investigate the effect of a perturbation of the local arrangement of polar heads on the micellar and mesomorphic properties

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of surfactants in aqueous solutions, we have studied the effect of a perturbation of the spontaneous arrangement of the polar groups induced by chemically linking the surfactant molecules two by two through a polymethylene spacer of adjustable length attached to the polar heads. We therefore synthesized a series of bis(quaternary ammonium bromide) surfactants of the type:



referred to below as m - s - m , 2Br^- . Their critical micelle concentration (CMC) and micelle ionization degree (α) have been reported elsewhere⁵. We found that the changes of CMC and α with m are similar to those of monoquaternary ammonium bromide surfactants. The change of CMC with the spacer carbon number s is more complex, which we attributed both to a conformational change of the surfactant ion at low s , and to a progressive penetration of the spacer into the micelle hydrophobic core at $s \geq 10$. This last point is supported by surface tension measurements involving the 12- s -12, 2Br^- series⁶. Finally, cylindrical and lamellar mesophases were observed in 12- s -12, 2Br^- /water mixtures⁷. In the case of the 12-8-12, 2Br^- homologue, the spacer lies flat at the interface, where it adopts a stretched conformation⁷.

This last result is important, as it indicates that the presence of the spacer can strongly affect the distribution of distances between polar heads. Spacers of intermediate s -values, say

between 4 and 8, correspond to distances between polar heads from about 0.6 nm to 1.1 nm, which is close to the average distance between polar heads in spherical or spheroidal micelles. Thus no special behaviour of the systems is expected in this range of s values. Indeed, the values of the micelle aggregation number, N , suggest that the micelles of the 12-4-12, 2Br^- and 12-6-12, 2Br^- homologues are nearly spherical. (Plots of N versus surfactant concentration, C , are nearly horizontal, and the values of N are very similar to those for spherical micelles (R.Z. and M. Benrraou, unpublished observations).) More interesting behaviour is expected for the $s=2$ and 3 homologues. The distances between polar heads are now locally much smaller. This affects the spontaneous curvature of the surfactant layer and, in turn, the shape of the micelles formed by these surfactants². Aggregation number measurements show rapid micellar growth upon increasing 12-3-12, 2Br^- concentration. No measurements have been made on the 12-2-12, 2Br^- homologue, but visual inspection (motion of air bubbles trapped in the solution) indicated that its solutions were viscoelastic, even at a concentration as low as 0.02 M (R.Z. and M. Benrraou, unpublished observations).

We have investigated the microstructure of 12- s -12, 2Br^- surfactants having $s=2, 3$ and 4 in aqueous solution. We used cryogenic transmission electron microscopy (cryo-TEM), a technique that can provide information on organized assemblies present in complex fluids such as viscoelastic micellar solutions, mixed lecithin-surfactant systems, anionic-cationic surfactant mixtures, and solutions of polyamphiphiles⁸⁻¹⁷. Cryo-TEM avoids most pitfalls encountered in studies of surfactant systems

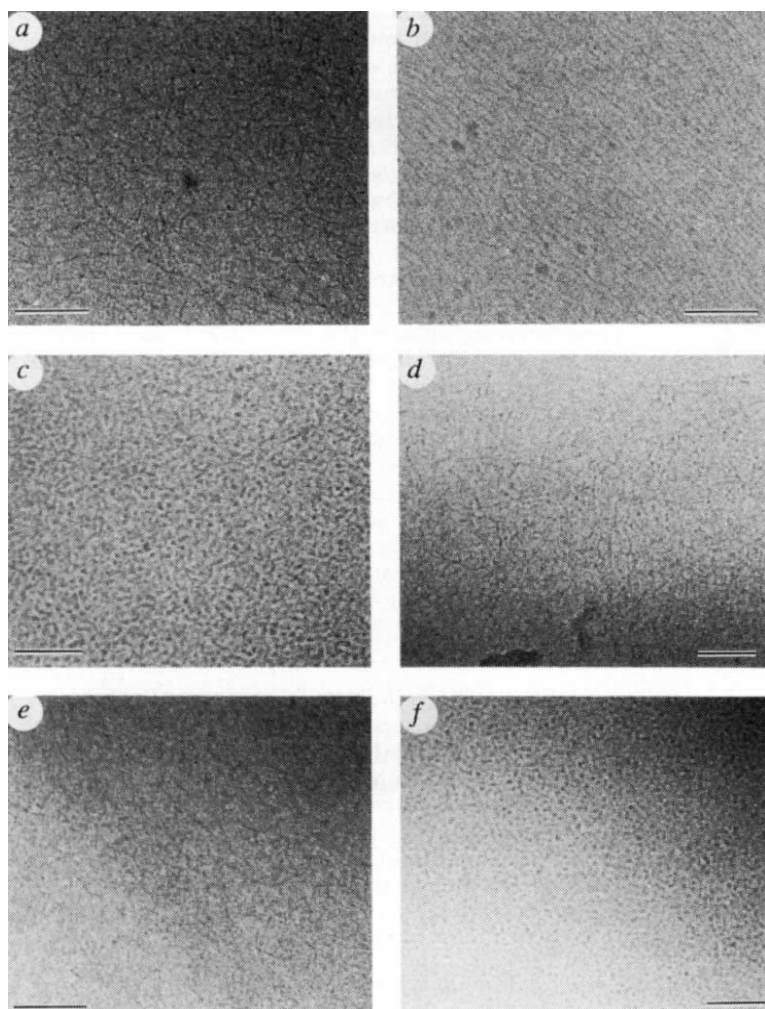


FIG. 1 Cryo-transmission electron micrographs of vitrified aqueous solutions of 12- s -12, 2Br^- dimeric surfactants. *a*, 2% solution of 12-2-12, 2Br^- showing strongly entangled thread-like micelles. *b*, 7% solution of 12-3-12, 2Br^- , in which the thread-like micelles are shorter than in the $s=2$ case, and have aligned as a result of flows in the liquid specimen before vitrification. *c*, Densely packed area of spheroidal micelles in a 5% solution of 12-4-12, 2Br^- . *d-f*, Solution of 12-2-12, 2Br^- /DTAB mixtures (1.7%) with increasing mole fraction (X) of DTAB in the mixtures, showing the progressive disappearance of the thread-like micelles and appearance of spheroidal micelles as X increases from 0.065 (*d*), to 0.14 (*e*) and 0.30 (*f*). Scale bar, 100 nm.

by electron microscopy¹⁸. Cryo-TEM specimens were prepared as before⁹ at 25 °C and 100% relative humidity.

Figure 1a and b shows typical electron micrographs of aqueous solutions of 12-2-12, 2Br⁻ and 12-3-12, 2Br⁻. The long, thread-like and entangled micelles seen in the micrographs explain the viscosity and viscoelasticity of the systems. These micrographs resemble those of aqueous solutions of cetyltrimethylammonium bromide (CTAB) in the presence of KBr (ref. 8), or of more dilute cetyltrimethylammonium chloride (CTAC) solutions in the presence of sodium salicylate^{10,15}.

Formally, the 12-2-12, 2Br⁻ surfactant may be considered as the dimer of the surfactant dodecyltrimethylammonium bromide (DTAB). Quasi-elastic light-scattering showed that DTAB micelles start growing only at extremely high concentration or in the presence of a high concentration of salt^{19,20}. The same is true for the longer homologue CTAB, for which micelle growth is detected only at concentrations above 0.2 M in the absence of salt²¹, or at lower surfactant concentration in the presence of NaBr or KBr (refs 19, 22). Thus in the case of DTAB, dimerization to the 12-2-12, 2Br⁻ surfactant enhances the tendency to form thread-like micelles and, in turn, dramatically alters the rheological behaviour of the system. This also means that the dimerization of the surfactant has increased its packing parameter from a value below 1/3, characteristic of spherical micelle-forming surfactants, to above 1/3, as for cylindrical micelle-forming surfactants².

Figure 1c shows a micrograph of an aqueous solution of 12-4-12, 2Br⁻. The thread-like micelles are no longer present. Spheroidal micelles are seen instead, as predicted from our discussion and from aggregation number measurements (R.Z. and Benraou, unpublished observations).

Finally, we examined three DTAB/12-2-12, 2Br⁻ mixtures with increasing DTAB mole fraction (X) in the surfactant mixture, when the addition of a spherical micelle-forming surfactant to a thread-like micelle-forming surfactant would be expected progressively to inhibit the capacity of the latter to form such micelles should the two surfactants co-micellize. At $X = 0.065$, the system still contains long and entangled thread-like micelles (Fig. 1d). At $X = 0.14$, micelles are still thread-like (Fig. 1a) but much shorter than at $X = 0.065$. At $X = 0.30$ (Fig. 1f), only spherical micelles are present. The viscoelasticity had already disappeared at the lowest value of X . These results indicate that DTAB and 12-2-12, 2Br⁻ do co-micellize, and that DTAB has the expected effect on the thread-like micelles of 12-2-12, 2Br⁻.

We have presented here some unusual effects associated with dimeric surfactants of the type $C_5H_{25}-\alpha,\omega-(C_{12}H_{25}N^+(CH_3)_2Br^-)_2$ with short C_5H_{25} spacers; most importantly, there is an enormously enhanced tendency to micelle growth which leads to viscoelasticity. Further investigation will be necessary to determine the range of validity of our results. They raise the question: if a monomeric surfactant forms thread-like micelles, would the dimeric surfactant derived from it form organized assemblies having the structure immediately after elongated micelles in the hierarchy of structures determined by packing parameter, that is disc-like micelles and vesicles? If so, a new approach to the spontaneous formation of vesicles will be possible. □

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Visualization of convective fluid flow in a porous medium

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WHEN a horizontal layer of fluid is heated from below, it may undergo Rayleigh-Bénard convection (RBC), leading to the spontaneous appearance of regular patterns of fluid flow¹. The shadowgraph technique², which allows visualization of the convection patterns, has assisted in developing an understanding of RBC. Related to RBC is convection in a fluid permeating a porous medium (called Horton-Rodgers-Lapwood convection or HRLC) when it is heated from below³⁻⁷. HRLC is relevant to geothermal applications and to flow in soils. Pattern formation in HRLC is less easily visualized by shadowgraph techniques because of the difficulties of transmitting light through the porous medium. Here we show how these difficulties can be overcome by constructing porous media in which the interfaces between solid and liquid are either parallel or perpendicular to the confining boundaries of the experimental system. Convection in such a medium can be visualized using conventional shadowgraph methods, and we compare the stationary flow patterns observed against measurements of heat transport.

Important issues for both RBC and HRLC include convective pattern selection, the influence of sidewalls and defects, heat transport and instability of the convection rolls. Sidewall forcing, defect dynamics and large-scale flows are important in pattern selection for RBC; consequently, straight parallel rolls are seen in this system only in special circumstances. Here we address some of the corresponding issues in HRLC; our experiments also reveal effects which do not have analogues in RBC, particularly the role played by the geometry of the medium.

HRLC, in the form of convection rolls, occurs⁶ in an isotropic horizontally infinite layer when the porous Rayleigh number (Ra)

$$Ra = \alpha g \gamma d \Delta T / \nu \kappa \quad (1)$$

exceeds $4\pi^2$. Here, α is the thermal expansion coefficient, γ is the permeability of the medium, ν is the kinematic viscosity, κ is an effective thermal diffusivity of the fluid and solid composite and g is the acceleration due to gravity. The temperature difference driving the convection is ΔT . The wavelength of the rolls is $\lambda = 2d$, where d is the height of the fluid layer; correspondingly, the dimensionless wavenumber $q = 2d\pi/\lambda$ is π at the onset of convection. An additional parameter of interest^{8,9} is the Prandtl number

$$Pr = \nu / \kappa \quad (2)$$

which is 3.8 for our media, saturated with deionized water. Although the Prandtl number plays no role in darcian flow (that

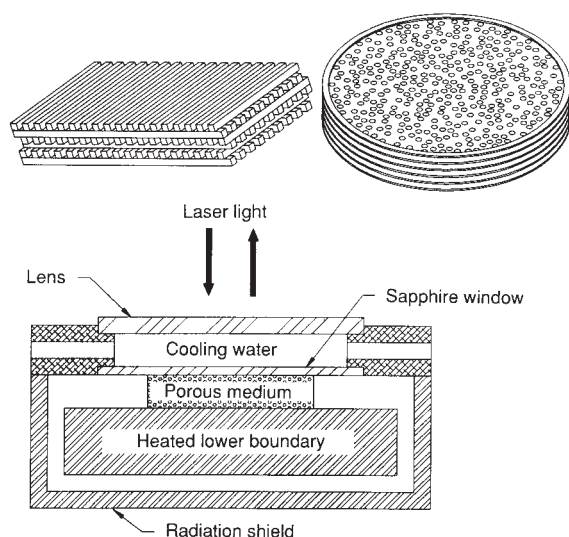


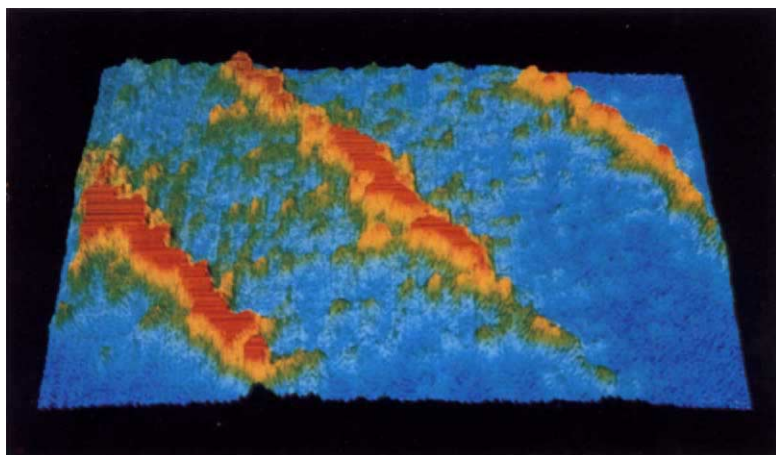
FIG. 1 Schematic diagrams of the rectangular porous medium (top left), the random medium (top right) and the shadowgraph apparatus (bottom). The overall dimensions of the rectangular medium are $d=0.902$ cm high, $X=5.56$ cm deep and $Y=2.70$ cm wide, giving aspect ratios $L_x=X/d=6.16$ and $L_y=Y/d=2.99$. The perforations in each disk of the random medium consist of 362 randomly placed, non-overlapping, 0.159-cm diameter holes. The aspect ratio (radius/ d) of the random medium is $L=3.05$ where $d=1.056$ cm.

is, very near onset), it does influence flow at higher Ra, which includes inertial effects.

In a typical experiment, a porous medium is constructed by partially filling a space with solid material^{10–15}, often spheres. These may be nominally randomly densely packed or, in some cases^{10,15}, regularly packed. The difficulty has been that even transparent solids refract light. Because there are generally many curved fluid–solid interfaces for a medium constructed from spheres, light paths are quickly randomized, and flow imaging is impossible. Refractive-index matching cannot be used because of the vertical temperature gradient. Visualization with other methods of imaging have been used, but in all cases of which we are aware, the technique has either been invasive^{13,14} or has provided only a side view¹². Local probes can provide useful information^{4,14}, but only a modest number of probes can be used.

The first medium that we used (the rectangular medium) consists of a grid of rectangular bars (Fig. 1). Six layers of bars with square cross-section (0.150×0.150 cm) are stacked with alternating orientations. Because the spacing between the bars is identical to the width of a bar, the porosity is 50%.

FIG. 2 Shadowgraph image near the onset of convection for the rectangular medium, using false colour to enhance the image. Red corresponds to bright regions (downflows) of the original image, and blue corresponds to dark regions (upflows). The temperature difference at the onset of convection is 4.07 K.



The second medium (the random medium), consists of five perforated disks, each 0.15 cm thick, sandwiched between 0.051 cm thick annular spacers. Two additional spacers separate the medium from the top and bottom plates. In this arrangement, the porosity is 31.4%

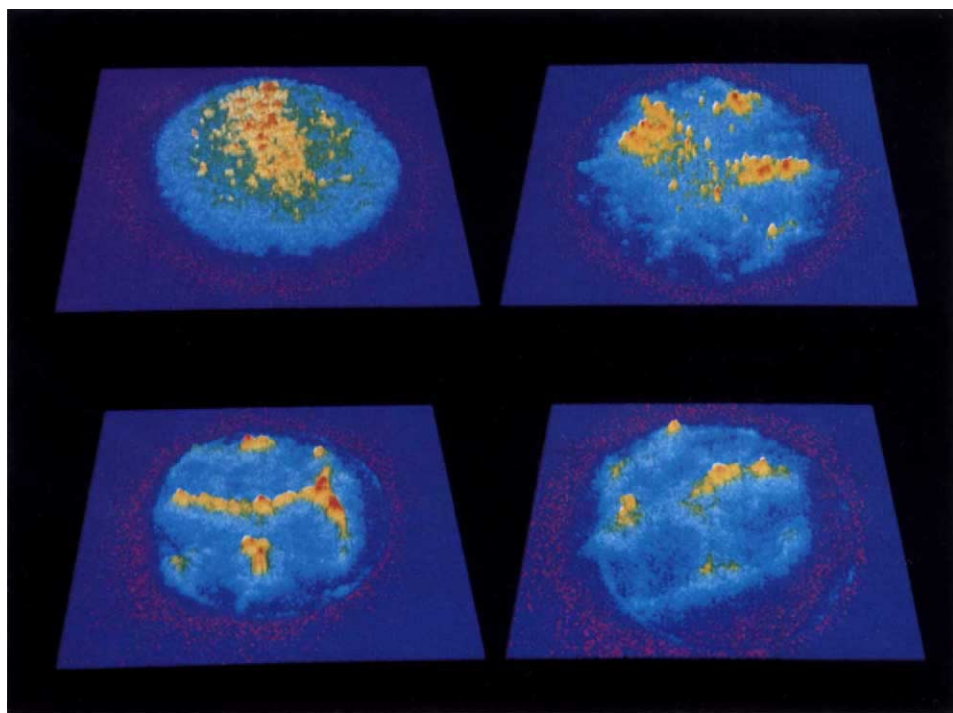
For each medium, γ and κ are different for the horizontal and vertical directions. They are essentially the same, however, in each of the horizontal directions. Additionally, the heights of both constructions are short enough to give vertical thermal relaxation times $t_r \equiv d^2/\kappa$ of about 10 min, so that meaningful experiments are possible on reasonable timescales.

The buoyancy-driven flows in our media are imaged¹⁶ by a conventional shadowgraph, as sketched in Fig. 1. Here, a broad collimated light beam passes through a sapphire window comprising the upper boundary, then through the fluid-saturated medium from the above. It is reflected from the optically flat reflecting metal surface forming the bottom plate, and traverses the medium a second time. The light is refracted by convection-induced gradients in the index of refraction. An image of the convection pattern then shows strong upflows as dark regions and strong downflows as bright regions. Sample shadowgraphs using false colour (red, brightest; blue, darkest) are given in Figs 2 and 3.

We first consider Fig. 2, which shows an image of the convective flow near onset for the rectangular case. Rolls form very near onset, but are oriented at 45° to the sides of the container. This contrasts with RBC, where, in a comparable geometry, rolls tend to be aligned parallel to the short sidewall¹⁷. Two effects are important. First, the velocity boundary condition at a wall within a porous medium is free-slip, whereas for RBC it is non-slip. In HRLC, therefore, wall forcing of the pattern may be less important than in RBC. Second, because an even number of layers are used, the perpendicular orientation of the top and bottom layers biases the system towards a diagonal orientation. The fluid must attain a pattern that has a horizontal velocity component aligned with the grid at the top and bottom layers, otherwise flow will be suppressed in these layers. The difference in the wavenumber $q = 3.6 \pm 0.3$ from $q = \pi$ may be explained by the difference in γ and κ in the horizontal and vertical directions¹⁸.

We next turn to images of convection in the random geometry, Fig. 3. Here, complicated cellular patterns typically occur near onset. Repeated cyclings through the onset of convection yield different convection patterns each time. We estimate that, for the cellular pattern, $q = 3.0 \pm 0.4$, which is consistent with expectations. There does not seem to be strong forcing of the pattern at the sidewalls. In particular, the pattern selected near onset seems to be determined randomly rather than by the geometry. One possibility is that although small, the local variations in the permeability and diffusivity (which appear in the Rayleigh number) may pin the defects. The effect of spatial

FIG. 3 Shadowgraph images near the onset of convection for the random cylindrical medium. False colour is used to enhance the image. Each image represents a separate cycling through the onset of convection which occurs at ~ 7.91 K.



porosity variations on pattern selection has been considered by Vincourt¹⁹. Although several convection patterns may be observed for the same container with RBC, flows in small aspect ratios tend to be limited to few patterns (with small variations) which are either axisymmetric or are roughly parallel rolls intersecting the sidewalls normally^{20,21}.

Once formed, the patterns, either of parallel rolls or random structures in the respective media, remain stable. They are steady from the critical porous Rayleigh number (Ra_c) to a porous Rayleigh number (Ra_t) of at least $2Ra_c$ to $3Ra_c$. Above Ra_t , the flows become time-dependent¹⁶.

The net heat transport¹⁶, with a resolution $\sim 0.1\%$, may be expressed as a Nusselt number, Nu (that is, the heat transport at a given Ra normalized by the conductive portion as shown in Fig. 4). Thus $Nu = 1$ for $Ra < Ra_c$, then Nu rises continuously

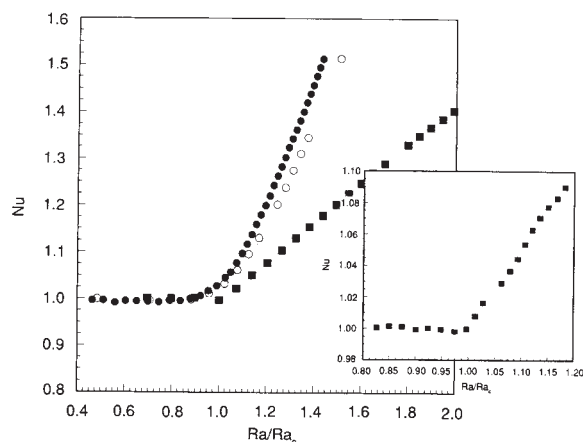


FIG. 4 Nusselt number (Nu) versus Ra/Ra_c for the random circular medium (open and closed circles, corresponding to two different runs) and the regular rectangular medium (squares). The inset shows data for the rectangular case on a finer scale to emphasize the sharpness of the transition to convection.

above unity for Ra increasing above Ra_c . Data for the cylindrical cases show that the various patterns have different Nu . In addition, there is some obvious rounding near onset. The rounding also may be symptomatic of spatial variations in γ and κ . By contrast, the results for the rectangular case show no rounding at this resolution.

Our designs open new avenues for the study of convection in porous media. Convection of both pure fluids and multicomponent mixtures is of interest¹⁵. Furthermore, these experiments show that the structure of the medium plays a role which does not have a simple analogue in conventional RBC. Once generated, a particular state remains frozen over a substantial range of Ra . The large number of different complex states seen in the random medium, and the diagonally oriented rolls in the rectangular medium, imply that the wall forcing mechanism which favours parallel rolls in RBC¹⁷ is less important in porous media convection. $\square$

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Climate-driven variations in geothermal activity in the northern Kenya rift valley

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HIGH-TEMPERATURE continental geothermal systems are primarily associated with volcanic activity at plate margins¹. It has long been known that the activity of these geothermal systems is intermittent, and this is generally attributed to the effect of magmatic intrusions or fault motions. Recently, however, it has also been recognized²⁻⁴ that surface-water hydrology and climate variations can influence the evolution of individual silicic caldera-hosted geothermal systems, although it is difficult to judge the general importance of such effects in controlling the long-term behaviour of continental geothermal systems. Here we report uranium-series ages for the hydrothermal deposits of past geothermal activity from several Quaternary volcanic centres in the northern Kenya rift valley. We find that the ages correspond well to periods of high lake level within the rift, suggesting that the elevated water table and increased availability of meteoric water associated with more humid climates can promote greater transfer of heat and mass from deep, long-lived heat sources to the surface.

In the northern Kenya rift valley, geothermal activity is associated with a series of Quaternary volcanoes (Fig. 1). Hot, hydrothermally altered ground and relatively weak fumaroles, but no active hot springs, are found on these volcanoes. On the intervening rift floor are hot springs representing deeply circulated groundwaters not involved significantly with the high enthalpy systems of the volcanoes⁵. The lack of hot springs and the weak nature of fumaroles on the volcanoes reflect deep water tables, a consequence of the semi-arid to arid climate of the region.

Former geothermal discharge areas have recently been recognized on the flanks and summits of several of these volcanoes. These are manifested by hot-spring sinters, silica veins and associated hydrothermal alteration. This former hot-spring activity at high elevations on the volcanoes could indicate that the region was more geothermally active in the past; the activity may have declined as a result of waning heat sources. However, gas geothermometry on fumaroles indicates high temperatures (~300 °C) at depth in some of the geothermal systems⁵⁻⁷. Emuruangogolak and the Barrier volcanoes have erupted within the past 100 years, suggesting that magmatic heat sources are still available to drive the geothermal systems. An alternative explanation for the presence of geothermal silica deposits high on the volcanoes is that deposition occurred when water tables were considerably higher during humid climates. High water tables would have been conducive to hot springs at elevated locations, without requiring changes in the intensity of heat sources. To understand better the geothermal history of the region, we collected samples of geothermal silica (Fig. 1) for age determination by uranium-series (U-series) methods (Table 1).

A striking result of these age determinations is the correlation between the times of hot-spring activity at elevated locations on volcanoes and high lake levels within the rift during the past 200 kyr (200,000 years). Evidence for high lake levels in the past consists of old shorelines associated with skeletal remains of aquatic organisms and littoral stromatolites, and subaqueous volcanic products (such as pillow-lava/hyaloclastite sequences intercalated with diatomites) on the flanks of several of the volcanoes. Figure 2 shows a compilation of age determinations

indicating times of high lake levels for the Kenya rift valley in comparison with results from the present study.

Three lakes within the study area fluctuated in size and depth during the late Quaternary. These are Lake Baringo in the south, Lake Turkana in the north, and the former Lake Suguta which occupied the inner trough of the rift between Emuruangogolak and the Barrier volcanoes at various times⁸. At one stage (~12–7 kyr ago) Lake Suguta covered an area of ~2,000 km² and reached depths of more than 300 m, but it has since desiccated in response to an increasingly arid climate⁸.

Sinter deposits associated with active fumaroles around the western rim of the caldera of the Barrier volcano have U-series ages ranging from 6.9 ± 0.3 kyr to 9.3 ± 0.5 kyr. These ages correlate with the period when Lakes Turkana and Suguta reached maximum levels on the flanks of the Barrier volcano. Lake Turkana fluctuated around a maximum level between 9.5 kyr and 7.5 kyr ago⁹, and the highest shoreline on Lake Suguta has a radiocarbon age of 9.7 ± 0.2 kyr⁸. Silica veins near active fumaroles along the Nakoporon fault zone on the western flanks of Korosi volcano have U-series ages of 11.9 ± 0.6 kyr and 12.7 ± 0.6 kyr. Lake Baringo, adjacent to Korosi, reached its highest recorded stand at 985 m elevation between about 12 kyr and 11 kyr¹⁰, when the nearby silica veins (1,000 m elevation) were deposited at Nakoporon. These deposits on the Barrier and Korosi volcanoes were contemporaneous with very high lake levels that occurred throughout the Kenya rift between about 12 kyr and 7 kyr ago⁸⁻¹², and correspond to a humid climate associated with the latest glacial-to-interglacial transition of stages 2 to 1 in the marine ¹⁸O record.

Sinter deposits associated with active fumaroles on the caldera rim of Emuruangogolak volcano have a U-series isochron age

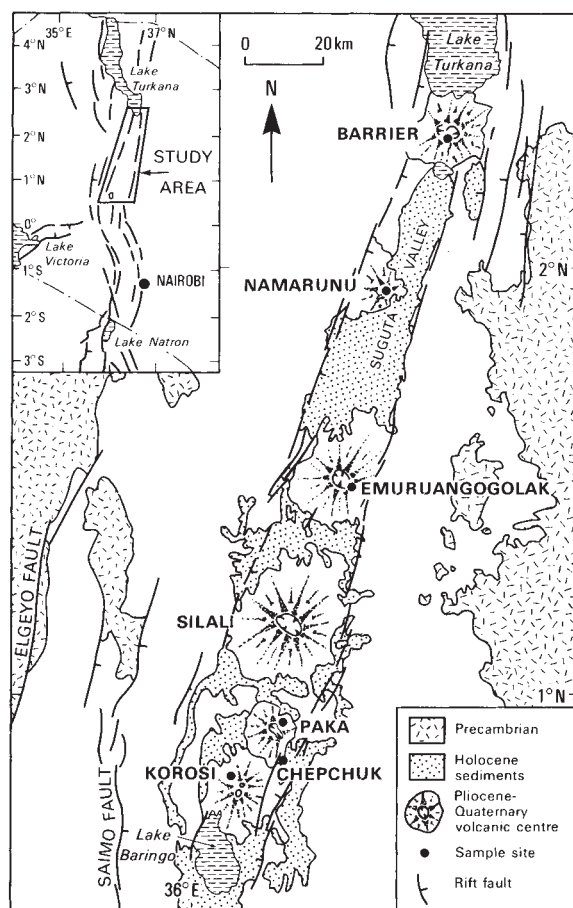


FIG. 1 Map showing location of sampled localities within the inner trough of the northern Kenya rift valley.

High lake levels

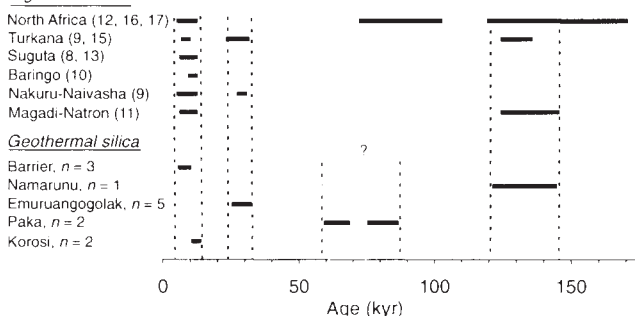


FIG. 2 Diagram showing U-series ages of geothermal silica deposits (this work) compared to periods of high lake levels in the Kenya rift valley and northern Africa during the past 200 kyr. Numbers in parentheses indicate references cited in text and n is the number of data points portrayed. Vertical dashed lines enclose periods of contemporaneous high lake levels and elevated geothermal activity on rift volcanoes.

of 29 ± 3 kyr. This falls within the period from about 35 kyr to 25 kyr when lakes in East Africa were again high^{9,13,14}.

Extensive silica deposits occur on the upper flanks and summit area of Namarunu. A massive vein from the upper eastern flank has a U-series age of 133 ± 11 kyr, contemporaneous with a period of high lake levels. A U-series age of 121 ± 20 kyr obtained from littoral stromatolites on a shoreline in the Namarunu area indicates that Lake Suguta reached high levels at this time¹³. High shorelines also occur in the southern part of the rift around the Magadi-Natron basin, where littoral stromatolites have U-series ages of 135 ± 10 kyr¹¹. There is also corroborative evidence for high stands in Lake Turkana around 130 kyr¹⁵. Hillaire-Marcel *et al.*¹¹ suggested that the high lake levels at 135 ± 10 kyr represent a humid climate during the glacial-interglacial transition of stages 6-5 in the marine ^{18}O record. Most of the Namarunu samples analysed are much older, but their high ($^{234}\text{U}/^{238}\text{U}$) values indicate ages < 2 Myr.

Silica veins associated with active fumaroles on the Lower Pleistocene volcanic centre of Chepchuk have poorly resolved U-series ages of 350 ± 60 kyr and 430 ± 120 kyr. These may cor-

respond to a period of high lake levels found elsewhere in the rift. Stromatolites related to former high lake stands in the Lake Magadi-Natron basin have uncorrected U-series ages of about 300 kyr, although a corrected age of 250 ± 40 kyr may be obtained by assuming that ^{234}U had been preferentially leached¹¹. This could represent a humid climate during the glacial-interglacial transition between stages 8 and 7 of the marine ^{18}O record¹¹.

Minor silica veinlets within faulted trachyte flows on the lower flanks of Paka volcano have U-series ages of 64 ± 4 kyr and 81 ± 5 kyr. These do not correlate with any known period of high lake levels in East Africa, although in northern Africa there is evidence for lake transgressions at around 90 kyr^{16,17}. This may be evidence for a previously unrecognized phase of humid climate in Kenya.

Only one episode of previous geothermal activity since 200 kyr has been dated at each volcano, whereas there are clearly at least three distinct periods of high lake levels. This is not a result of sampling bias on our part, as all known sites of geothermal silica deposits were sampled. Several geological factors may explain this apparent discrepancy. First, evidence of additional periods of hot spring activity on each volcano may have been obscured by a cover of younger volcanic products. For example, the flanks of Emuruangogolak and the Barrier are virtually covered by lavas post-dating the 120–140-kyr high lake stand, and both volcanoes have extensive cover of subaerial basalts and trachytes that even post-date the 10-kyr high lake stand. Second, the region is tectonically active, and substantial faulting and erosion have occurred throughout the Pleistocene. Taking these factors into account, we believe that the correlation of periods of humid climate with the ages of preserved geothermal silica deposits in the northern Kenya rift valley is significant. □

TABLE 1 U-series data and calculated ages for geothermal silica samples

Sample	U ($\mu\text{g kg}^{-1}$)	($^{234}\text{U}/^{238}\text{U}$)	($^{230}\text{Th}/^{232}\text{Th}$)	($^{230}\text{Th}/^{234}\text{U}$)	Age, kyr
BAR-1-A	1,260 (60)	1.07 (0.02)	40 (5)	0.061 (0.004)	6.9 (0.4)
BAR-1-C	1,060 (40)	1.06 (0.02)	46 (5)	0.082 (0.005)	9.3 (0.5)
BAR-1-F	1,710 (20)	1.07 (0.01)	50 (10)	0.068 (0.003)	7.6 (0.3)
NAM-2-A	350 (10)	1.06 (0.02)	220 (20)	0.71 (0.03)	133 (11)
NAM-2-B	310 (10)	1.40 (0.03)	$\geq 17,000$	1.14 (0.06)	≥ 380
NAM-2-C	271 (8)	1.63 (0.03)	1,280 (350)	1.20 (0.06)	≥ 440
NAM-2-D	419 (6)	1.36 (0.02)	$\geq 14,000$	1.14 (0.03)	≥ 670
EMU-3-A	280 (10)	1.01 (0.04)	4.4 (0.3)	0.28 (0.02)	5-point isochron 29 (3)
EMU-3-C	530 (10)	1.00 (0.01)	7.1 (0.4)	0.27 (0.01)	
EMU-3-D	265 (4)	1.04 (0.02)	1.71 (0.06)	0.43 (0.02)	
EMU-3-E	179 (3)	1.04 (0.03)	3.8 (0.2)	0.40 (0.02)	
EMU-3-F	442 (5)	1.04 (0.01)	5.9 (0.5)	0.29 (0.01)	81 (5)
PAK-1-A	570 (10)	1.01 (0.02)	370 (90)	0.52 (0.02)	
PAK-1-B	410 (10)	1.01 (0.03)	430 (150)	0.45 (0.02)	64 (4)
CHEP-2-A	370 (10)	1.29 (0.04)	27 (2)	1.02 (0.03)	350 (60)
CHEP-2-B	249 (9)	1.10 (0.05)	13 (1)	1.02 (0.03)	430 (120)
KOR-1-A	1,110 (30)	1.00 (0.02)	22 (2)	0.104 (0.005)	11.9 (0.6)
KOR-1-B	1,220 (30)	0.99 (0.02)	16 (2)	0.110 (0.005)	12.7 (0.6)

Errors ($\pm 1\sigma$, given in parentheses) based on statistics of α -counting method¹⁸. Decay constants used for age calculations: ^{230}Th , $9.195 \times 10^{-6} \text{ yr}^{-1}$; ^{234}U , $2.835 \times 10^{-6} \text{ yr}^{-1}$; ^{238}U , $1.551 \times 10^{-10} \text{ yr}^{-1}$. Abbreviations used for sample locations as follows. BAR, Barrier (Kakorinya) volcano. Sinter float and veins on upper southwest wall and rim of caldera, associated with faults and fractures cutting a small trachyte dome. Pervasive alteration, brecciation and fumarolic activity (elevation 910 m). NAM, Namarunu volcano. Silica veins from summit area east of main fault scarp, above area of hydrothermal alteration (elevation 720 m). EMU, Emuruangogolak volcano. Area of sinter float and layered veins on rim and southwest-facing slope of caldera wall, associated with clay alteration and fumarolic activity (elevation 1,140 m). PAK, Paka volcano. Silica float blocks from low, north-south ridge on N flank, associated with fault cutting trachyte lavas (elevation 1,200 m). CHEP, Chepchuk volcano. North-south ridge, east of Nagoreti fault, on northwest corner of former caldera structure, southwest of fumarolic activity. Massive chalcidonic veins and float blocks (elevation 1,100 m). KOR, Korosi volcano. Silica vein cutting Nakoporon fault scarp, northwest flank of volcano, associated with fumarolic activity (elevation 1,000 m).

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Evidence from earthquakes for bookshelf faulting at large non-transform ridge offsets

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MIGRATING non-transform offsets, which occur along mid-ocean ridges when a propagating segment gradually elongates and takes over spreading from a neighbouring 'doomed' rift segment¹⁻³, represent a significant modification of the plate tectonic paradigm⁴. The migrating offset zone between the two spreading segments is clearly a locus of significant deformation, but the mechanism of this deformation has been controversial. Here we use the source parameters and locations of earthquakes at such offsets to discriminate between previously proposed models^{2,4-7}. Earthquakes at large non-transform offsets at medium and fast spreading rates show strike-slip mechanisms, with nodal planes rotated relative to the expected transform fault orientation. One set of nodal planes (presumably corresponding to the fault planes) is parallel to curved sea-floor lineaments which show increased rotation as a function of position in the deforming zone. A bookshelf faulting model in which initially ridge-parallel faults are rotated by simple shear is consistent with the observed lineament orientations, focal mechanisms and earthquake distributions.

Four previously proposed models of tectonics at migrating non-transform offsets (propagators) make different predictions about seismicity (Fig. 1). The instantaneous transform model consists of a single fault joining the two spreading axes^{2,5} (Fig. 1a). This narrow zone of deformation migrates as the propagating limb extends. The transform fault would produce a narrow band of strike-slip earthquakes with fault planes parallel to the plate motion direction.

The remaining three models describe a wide region of deformation between the spreading segments, with earthquakes expected throughout this zone. Each model, however, predicts different faulting mechanisms. For the two distributed transform models, a series of strike-slip transform faults joins the offset rifts^{2,4}. Faults in the transform parallel shear model are arranged parallel to plate motion and may be illustrated by a deck of cards with a shear couple parallel to the card faces (Fig. 1b). Faults in the transform oblique shear model⁴ are oriented oblique to plate motion, introducing a component of extension (Fig. 1c).

Bookshelf faulting consists of a series of faults initially oriented at a high angle to the shear couple, which are rotated as deformation proceeds⁶⁻¹⁰ (Fig. 1d). This model may be simulated by a bookshelf with a shear couple perpendicular to the book covers. At migrating ridge offsets the bookshelf faults originate parallel to the ridge axis and progressively rotate as a function of time and position in the deformation zone^{6,7}. This rotation results in strike-slip earthquakes with varying fault strikes, unlike the distributed transform models which predict the same strike for faults throughout the active region. Bookshelf faulting has been observed in rift environments on land in the South Iceland Seismic Zone^{11,12} and in southern Afar¹³.

We systematically studied teleseismic earthquakes associated with propagators and other large non-transform offsets along medium and fast spreading rifts to constrain the mechanism of deformation between the ridge axes. Earthquakes are particularly important clues to the mechanics of non-transform offsets because, unlike seafloor lineaments and topography, they show current regions of deformation and directions of active

fault movement. Shallow earthquakes were relocated using a hypocentroidal decomposition algorithm¹⁴ that inverts P wave arrival times to yield accurate relative locations within a cluster of events. We investigated seismicity at more than 60 non-transform offsets, relocating earthquakes in only 10 areas as most offsets produced few teleseismic earthquakes.

Focal mechanisms were obtained by inverting long period and broadband P and SH seismograms from stations at teleseismic distances using synthetic seismograms calculated with ray theory¹⁵⁻¹⁷. For several small events, source parameters were determined by inverting selected portions of the body wavetrain observed at regional and teleseismic distances using synthetics calculated with a reflectivity method¹⁸⁻²⁰. Harvard centroid moment tensor solutions²¹ were compiled for several recent events which we have not yet analysed. Focal mechanisms could not be determined for many small events because of the poor signal to noise ratio.

We obtained source mechanisms for the 27 earthquakes listed in Table 1. Figure 2 shows the earthquake locations and focal mechanisms for the two largest and most seismically active non-transform offsets: the Lau Basin²² and 29° S East Pacific

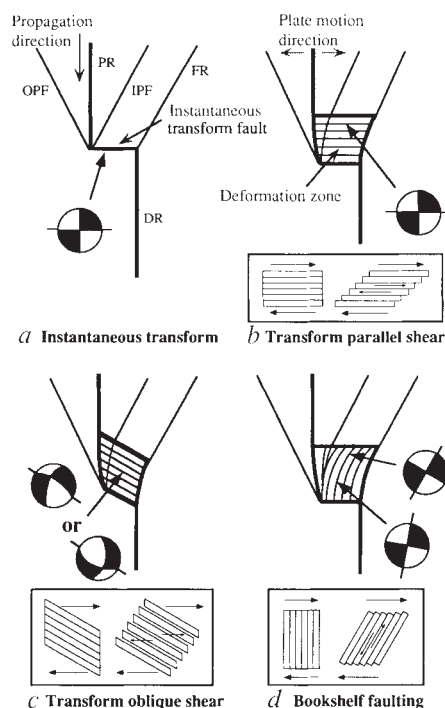


FIG. 1 Four models of propagating rift tectonics and the corresponding expected earthquake focal mechanisms. Tick marks projecting through a diameter of each focal mechanism identify the active fault plane. Insets are simple cartoons illustrating the relative motion of the fault blocks due to a right-lateral shear couple. **a**, An instantaneous transform is a single narrow fault zone parallel to plate motion that produces strike-slip focal mechanisms. **b**, A series of transform parallel faults generates strike-slip earthquakes on multiple faults parallel to the plate motion direction. **c**, A group of transform faults oblique to the shear couple produces earthquakes on multiple faults with components of both strike-slip and extensional dip-slip motion. **d**, In bookshelf faulting the orientations of the strike-slip faults vary with position, rotating clockwise as the left-offset propagating rift passes. A left-offset ridge discontinuity causes clockwise rotation and left-lateral slip along multiple bookshelf faults. Tectonic elements of the propagator are abbreviated as follows: PR, propagating rift; DR, doomed rift; FR, failed rift; OPF, outer pseudofault; and IPF, inner pseudofault. The pseudofaults show the historical trace of the propagator tip on each plate and separate younger sea floor created at the propagating rift from older crust generated at the doomed rift. The boldest lines mark the plate boundaries. Plate motion occurs perpendicular to the plate boundaries in all four models.

TABLE 1 Earthquake source mechanisms at large non-transform offsets

Location	Year	Day	Month	Latitude	Longitude	Strike*	Dip	Slip	km†	m_0	M_0 ‡	Ref.§	Qual.	Angle¶
Lau Basin ²²	1969	20	7	-19.36	-176.28	215	65	10		5.1	2.5	TS	B	25
~130 mm yr ⁻¹ ‡	1972	8	5	-19.54	-176.06	31	82	352	5	5.7	8.2	TS	A	21
	1976	7	12	-19.06	-176.47	55	70	354	8	5.5	13.0	TS	A	45
	1979	24	11	-18.97	-176.49	48	80	10	7	5.8	41.0	TS	A	38
	1987	9	1	-19.40	-176.45	200	75	10	8	5.9	68.0	TS	A	10
	1988	27	8	-19.58	176.23	25	76	337		5.2	5.1	TS	B	15
EPR 29° S ²³	1970	18	11	-28.80	-112.87	213	60	355	7	5.4	10.0	TS	A	28
158 mm yr ⁻¹	1976	23	7	-28.72	-112.68	35	85	335		5.2	2.3	TS	C	30
	1976	29	8	-29.80	-111.86	222	70	365		5.5	3.7	TS	C	37
	1977	15	7	-29.46	112.40	197	90	5		5.2	6.1	TS	C	12
	1982	23	8	-29.12	-111.93	38	87	345		4.9	1.8	CMT		33
	1982	9	12	-29.05	-112.56	195	85	350		5.5	10.0	TS	B	10
	1983	21	8	-28.81	-112.55	18	65	354		5.3	6.6	TS	B	13
	1983	31	8	-29.56	-111.84	35	90	0		5.2	1.4	CMT		30
	1991	3	1	-29.67	-111.95	224	82	347		5.4	7.7	CMT		39
Gal. 87° W ²⁸	1987	20	10	0.91	-87.09	119	54	350		5.2	2.8	CMT		29
64 mm yr ⁻¹	1991	9	10	0.82	-87.48	302	90	0		5.0	3.6	CMT		32
Gal. 95° W ^{6,24}	1978	5	3	2.63	-95.39	287	56	293		4.7	1.7	TS	C	—
51 mm yr ⁻¹	1989	17	2	2.72	-95.33	120	80	25		4.8	0.9	TS	C	27
	1989	13	12	2.63	-95.39	292	90	0		4.4	0.8	CMT		19
Gal. 99° W ²⁹	1987	27	2	2.26	-98.71	116	78	338		4.8	0.7	CMT		18
46 mm yr ⁻¹	1988	12	2	2.30	-98.81	297	90	11		5.3	1.9	CMT		19
Rivera-EPR ³⁰	1966	22	5	21.04	-108.82	250	50	22		5.2	1.5	TS	B	39
(three offsets)	1966	23	5	21.21	-108.76	240	55	10		5.2	2.3	TS	B	29
58–68 mm yr ⁻¹	1984	17	2	20.52	-109.09	55	70	10		5.3	10.0	TS	B	24
	1985	24	8	21.67	-108.49	251	81	3		5.1	2.9	TS	B	40
EPR 2.8° S ²⁶	1984	11	1	-2.51	-102.67	25	82	353		5.0	2.2	CMT		15
135 mm yr ⁻¹														

* Plane chosen which would correspond to the bookshelf faulting model.

† Depth in kilometres beneath the sea floor. Depths are given only for the best resolved earthquakes; other events can be fitted equally well for depths ranging between 3 and 9 km.

‡ Seismic moment in 1×10^{17} N m.

§ TS, results from this study. CMT, results from Harvard centroid moment tensor solutions²¹.

|| Approximate uncertainty in fault parameters: A, $\pm 5^\circ$ to 10° ; B, $\pm 10^\circ$ to 15° ; C, $\pm 15^\circ$ to 25° , with fault strike generally better constrained than dip.

¶ Angle of obliquity measured between the ridge orientation and the nodal plane corresponding to bookshelf faulting. (Measured clockwise for left offsets.) One Galapagos (Gal.) 95° W earthquake is a normal faulting event on the spreading centre for which we did not calculate a rotation.

‡ Full spreading rates calculated using the NUVEL-1 plate motion model^{31,32} except for the Lau Basin rate which was estimated using preliminary Global Positioning System measurements³³.

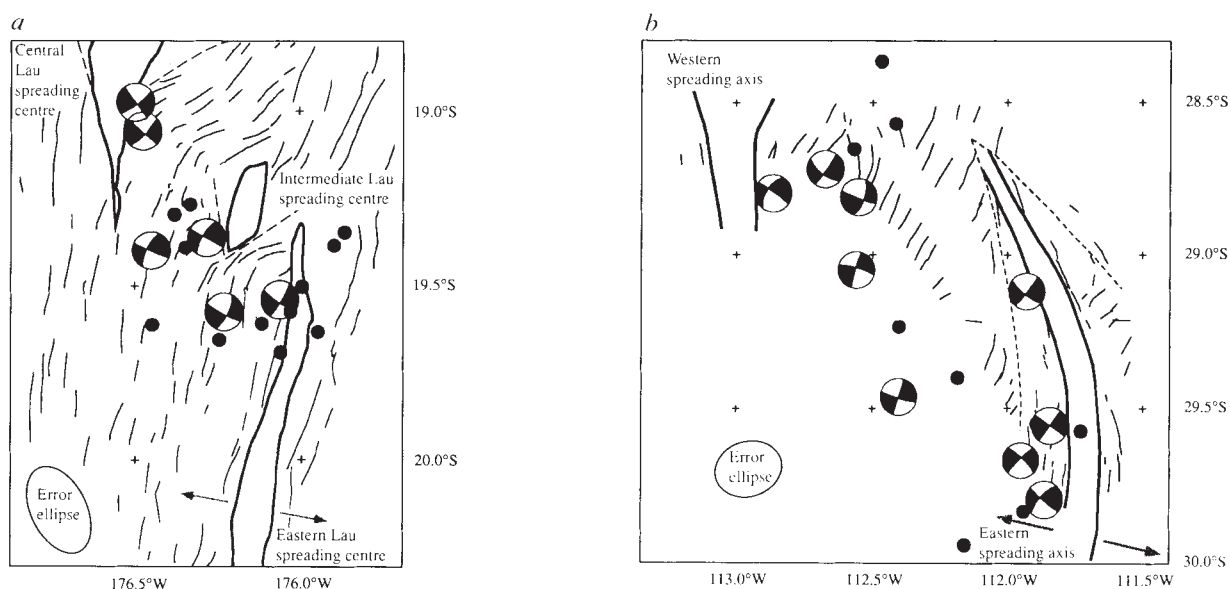
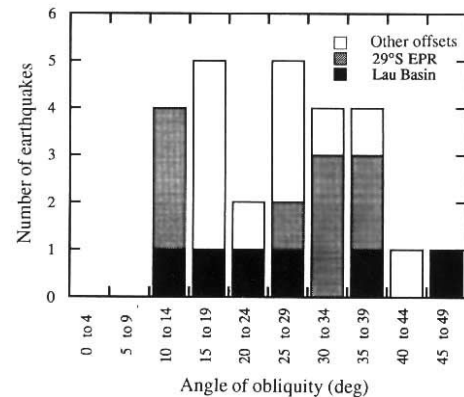


FIG. 2 *a*, Central Lau Basin propagator with earthquake locations and focal mechanisms determined in this study. The rift axes, selected lineaments, and pseudofaults (dashed) are as mapped with GLORIA sidescan sonar²². The Central Lau spreading centre is propagating south as spreading ceases along the Eastern Lau spreading centre. *b*, Earthquake locations and focal mechanisms at the 29° S East Pacific Rise offset, along with selected lineaments mapped in a limited region using SeaMARC II sidescan sonar²³. The eastern spreading centre is currently propagating northward. Arrows at the lower right of each figure show the approximate spreading direction.

The ellipse at the lower left represents the average 95% confidence region for each relative earthquake location. Earthquake mechanisms are listed in Table 1. In both regions northeast-striking earthquake nodal planes parallel curved seafloor lineaments, suggesting that strike-slip faults rotate clockwise as the rift propagates, in accord with the bookshelf faulting model for left offsets. Lineaments oriented at large angles to the ridge axes, expected for the other models of migrating non-transform offset deformation, are absent.

FIG. 3 Angles between the observed earthquake nodal plane and the ridge orientation for the strike-slip earthquakes in Table 1. The nodal plane and sense of rotation measured are the ones appropriate for a bookshelf faulting model (clockwise rotation and left-lateral faulting for a left-offset discontinuity). All earthquakes show the sense of rotation expected for bookshelf faulting. The fault orientations show a continuous distribution as expected for the bookshelf faulting model and suggest that originally ridge-parallel faults are rotated through angles of up to 45° .



Rise²³ offsets. The relocated seismicity occurs over a wide area along axis, clearly precluding an instantaneous transform model. Earthquake focal mechanisms are rotated relative to the expected transform fault geometry, but the seismicity data are unable to distinguish which nodal plane represents the actual fault plane. In most cases, however, there is agreement between a nodal plane and seafloor lineament orientations. For example, in the Lau Basin northeast-striking nodal planes correspond to mapped seafloor lineaments²² (Fig. 2a), suggesting that the earthquakes occur along northeast-striking left-lateral faults.

The observed earthquakes provide strong support for the bookshelf faulting model (Figs. 1 and 2). Northeast-striking nodal planes at the Lau propagator are compatible with bookshelf faulting, which predicts clockwise rotation of initially ridge-parallel faults for a left-offset discontinuity. The nodal planes strike more easterly in the northern part of the deformation zone, suggesting a systematic clockwise rotation of the fault planes as the rift propagates southward, consistent with the curved seafloor fabric. Seismicity is concentrated where lineaments show the greatest curvature, as expected for the bookshelf model, because slip occurs only where faults are being rotated.

The earthquake mechanisms are incompatible with the instantaneous and distributed transform models. The earthquake nodal planes are not oriented in the direction of relative plate motion as predicted by the transform parallel shear model, and generally do not show a significant extensional component, as predicted by the transform oblique shear model. The Lau Basin²², 29° S East Pacific Rise²³ and 95° W Galapagos^{6,24,25} offsets also show a complete absence of lineaments at large angles to the ridge orientation which could represent the transform faults predicted by these models.

Earthquakes at other large non-transform offsets (Table 1), including several offsets lacking a consistent migration direction, also show strike-slip focal mechanisms with nodal planes rotated relative to the expected transform fault orientation. These

earthquakes are strikingly similar to those associated with the Lau Basin and 29° S offsets. At the 2.8° S East Pacific Rise offset, for example, the focal mechanism is rotated 15° clockwise relative to the expected transform fault mechanism and has one nodal plane strike of 025 (N25° E), consistent with prominent lineaments orientated at 020 to 025 in the overlap zone²⁶. This suggests that bookshelf faulting may be the dominant mechanism of deformation at a variety of non-transform offset types.

Fault strikes obtained from earthquake focal mechanisms at large non-transform offsets suggest that bookshelf faults are continuously rotated from original orientations parallel to the spreading centres through angles of up to 45° (Fig. 3). A continuous distribution of fault orientations is observed, as would be expected for the bookshelf faulting model. This is incompatible with the other models which predict uniform fault orientations.

A previous explanation of bookshelf faulting at propagators⁷ suggested that the bookshelf faulting is occurring along reactivated abyssal hill normal faults, which should show dip angles of 45° to 60° (ref. 27). We find that the earthquakes generally show fault dip angles of greater than 70°. This suggests that the bookshelf faulting is occurring not along reactivated normal faults, but on throughgoing vertical faults with the initial strike controlled by the ridge-parallel seafloor fabric. In the South Iceland Seismic Zone the active bookshelf faults are vertical strike-slip faults which are not parallel to older, inactive rift faults (P. Einarsson, personal communication), implying that bookshelf faulting can occur through the creation of new faults.

Strike-slip earthquakes with fault planes oblique to plate motion constitute a new type of fault geometry for earthquakes along mid-ocean ridges, distinct from well-known strike-slip transform and ridge-parallel normal faulting. The present results suggest that bookshelf faulting is the dominant mode of lithospheric deformation within large non-transform offsets of medium- and fast-spreading ridges. □

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Mutual sexual selection in a monogamous seabird

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DARWIN¹ believed that elaborate ornamental traits expressed in both sexes might be favoured by mutual sexual selection driven by both female and male mate choice. Experimental studies on birds^{2–5} and fish^{6–9} have shown that male ornaments can be favoured by female mating preferences. But the concept of mutual mate choice has remained untested experimentally, although it has been supported by recent modelling¹⁰. Here we report the results of a study of mate preferences of the crested auklet *Aethia cristatella*, a monogamous seabird in which both sexes are ornamented. In two experiments we recorded the sexual response of male and female auklets to realistic opposite-sex models with crest ornaments experimentally shortened and lengthened within the range of natural variation. Males responded to accentuated female models with more frequent sexual displays, as did females to accentuated male models, confirming the idea that ornaments expressed in both sexes could be favoured by mutual mating preferences.

The crested auklet is a socially monogamous, sexually monomorphic seabird in which both sexes contribute parental care. During the breeding season, adults of both sexes have a spectacular forehead crest consisting of 2–23 (mean, 12) narrow forward-curving feathers, 8.1–58.5 mm in length (Fig. 1). The crest is displayed during courtship encounters at breeding colonies. Courtship occurs throughout the breeding season, as individuals pair before laying for breeding in the same year, or pair later in the season for breeding in the following year. Mate fidelity between breeding seasons is relatively low¹¹, so a high proportion of the population re-pair each year. But many individuals fail to pair early enough in the season to breed, and others fail to obtain mates¹². The resulting variation in mating success in both sexes provides an opportunity for sexual selection to favour ornaments that are displayed during courtship.

To test whether crest size could be favoured by male and female mating preferences, we performed two manipulation experiments using realistic models made from mounted skins of three male and three female crested auklets of average appearance. By manipulating ornaments on models we were able to eliminate two factors that can confound mate choice experiments involving manipulation of living birds: (1) intrasexual competition¹³, and (2) behavioural changes in a live bird resulting directly from manipulation of its ornament^{14,15}.

We tested for a mating preference for large crests by comparing the attractiveness of models with short crests to the same models with crest feathers lengthened (Fig. 1), using three

models of each sex to ensure that responses were not biased by unique characteristics of any one specimen. To lengthen the crest, we used cyanoacrylate 'superglue' to attach crest shafts collected from other birds to existing crest feathers on models. Manipulations involved changing the length of 10–12 shafts on each model to alter overall crest length and size while maintaining a natural appearance. Presentations were made on more than 30 different large display rocks (boulders with 2–12 m² flat upper surfaces, where auklets congregate to engage in courtship^{12,16}) throughout a colony of about 200,000 crested auklets at Buldir Island, Alaska (52° 2' N 175° 5' E)¹⁷. For each auklet that responded to a model, we identified its sex by bill shape, and recorded the occurrence of sexual displays, closest approach (0 cm, 1–10 cm, 11–20 cm, 21–30 cm, or >30 cm), and response duration (1–5 s, 6–10 s, 11–15 s, or >15 s). Response scoring was identical between two observers because these displays and measurements were simple and unambiguous. Because models were moved frequently from rock to rock throughout an immense colony, we believe no individual's response was scored more than once. This was supported by observations of approaches to models at a study plot where more than 400 colour-marked birds were present; no marked individual approached more than once. To control for intrasexual competition and interference among responding birds, we did not score approaches when more than one auklet was present near the model. Sham-manipulations were not performed because ornament manipulation could not have affected model 'behaviour', and there were no visible artefacts of manipulation. Crested auklets did not respond to natural looking least auklet *Aethia pusilla* models.

Crested auklets of both sexes approached the models as in natural courtship encounters, performing four types of stereotyped courtship display (arch, hunch, ruff-sniff and touch^{11,12,18,19}) to opposite sex models. In nature, these displays are performed only in courtship, increase in intensity with sexual attraction between courting auklets, and lead to the formation of mated pairs^{11,12,16}. In our model experiments, 29% of approaching females and 20% of approaching males performed a sexual display, other individuals merely looked at the model before departing. Models with enlarged crests were extremely attractive to approaching auklets of the opposite sex, compared with the same models with small crests. Females responded to male models with significantly more frequent arch, hunch and touch sexual displays when the models were presented with accentuated crests; males responded to female models with significantly more frequent ruff-sniff and touch displays (Table 1). The models were also approached more closely and for a longer duration when presented with large crests (female responses: for closest approach, log-likelihood ratio $G = 63.8$, d.f. = 4, $P < 0.0001$; for duration $G = 127.3$, d.f. = 3, $P < 0.0001$; male responses: for closest approach $G = 28.9$, d.f. = 4, $P < 0.0001$; for duration $G = 36.0$, d.f. = 3, $P < 0.0001$). Overall, these results indicate a strong relationship between sexual attractiveness and crest size in both sexes. Because larger crests are preferred, auklets bearing them are more likely to obtain mates or form pair bonds earlier, a suggestion supported by observations of the marked auklet population.

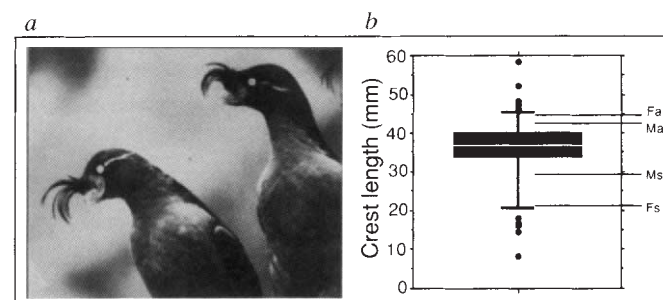


FIG. 1 a, Crested auklet courting pair. b, Variation in crest length among 253 adult crested auklets measured at Buldir. Box plot indicates 5th percentile (lower bar), 25th percentile (bottom of box), median (white line through box), 75th percentile (top of box) and 95th percentile (upper bar); outliers are represented by points. Crest size did not differ significantly between males and females in this population (based on birds sexed by behaviour or dissection: median male crest, 38.6 mm, $n = 32$; median female crest, 37.3 mm; $n = 56$; Mann-Whitney U ; $Z = 0.7$, $P = 0.5$; males were about 1.5% larger in measures of body size than females). The mean crest size of the models used in the experiments are indicated by: Fa accentuated female crests; Fs small female crests; Ma accentuated male crests; Ms small male crests (manipulated crest lengths were within 2 mm of the mean for each treatment). Female model crests were manipulated to a greater extent (24 mm) than males' (14 mm) because we suspected *a priori* that male mating preferences could be more difficult to detect.

TABLE 1 Responses to male and female ornament manipulations

Sexual display	Female response to male models					Male response to female models				
	Displays to big crest (151 birds)	Displays to small crest (269 birds)	Relative frequency big/small	G (d.f. = 1)	P	Displays to big crest (124 birds)	Displays to small crest (209 birds)	Relative frequency big/small	G (d.f. = 1)	P
Arch	28	2	24.9	47.2	<0.0001	6	8	1.3	0.2	NS
Hunch	31	7	7.9	36.3	<0.0001	13	17	1.3	0.6	NS
Ruff sniff	6	5	2.1	1.6	NS	16	11	2.5	5.9	0.01
Touch	24	19	2.3	7.6	0.008	9	3	5.1	7.4	0.006
One or more	76	29	4.7	77.8	<0.0001	45	35	2.2	15.9	<0.0001

Based on responses of 420 females (269 to small-crested male models and 151 to big-crested male models; scored by I.L.J.) and 333 males (209 to small-crested female models and 124 to big-crested female models; scored by F.M.H.) during 165 h of observation between 7 June and 14 July 1992. Each model was presented with a small crest, then with accentuated crest, then with small crest again on about 10 d each; roughly equal numbers of responses were scored in each phase. 'Relative frequency, big/small': relative frequency of display to accentuated versus small crested models (for example, females' arch displays were 24.9 times more frequent to the accentuated model). Sex was determined by bill shape: males have a larger and more strongly hooked bill than females (refs 18, 22, 23). There was no significant difference in response among models (for displays and response measures: female models, $G=2.0-4.0$, d.f.=1-8, $P=0.2-0.4$; male models, $G=0.1-2.4$, d.f.=1-8, $P=0.4-0.9$), so responses were pooled for all models within each experiment. Female responses to long-crested models were significantly stronger than males' responses for arch ($G=43.5$, d.f.=1, $P<0.0001$), hunch ($G=36.8$, d.f.=1, $P<0.0001$), and touch displays ($G=7.9$, d.f.=1, $P=0.004$). There was a slight seasonal effect of gradually declining response to models, but this could not have biased the results because the response to crest accentuation went in the opposite direction, increasing significantly between early (small crests) presentations and the first accentuated crest presentations. Median presentation dates were the same for small-crested and accentuated-crested models.

In our experiment, male responses to accentuated female models were less strong than female responses to male models (although both were significant; Table 1). This initially suggests that female preferences are more important, and raises the question of why female crested auklets have a similar sized crest ornament to males. One possibility is that the sexes have an equal role in mate choice, but our experiment underestimated the expression of male preference because the sexes differ in the way they initiate courtship. Male and female responses to the static models could have differed because crested auklet males tend to wait for females to approach, then aggressively reject unwanted partners, whereas females are more active in seeking out and approaching males (although both sexes show either type of behaviour)¹¹. At present we cannot quantify the relative strength of the preference; the solution to this question requires more detailed study of natural pair formation. In our experiment, we controlled for intermale and interfemale competition by excluding responses when more than one bird was near the model, but because crest size is also correlated with dominance in both sexes and aggressive competition for mates is frequent (I.L.J., unpublished data), crest ornament size is likely to be favoured by intrasexual competition as well as by mating preference.

Our experiments indicate that crested auklet ornament size is likely to be favoured by sexual selection involving mutual mate choice by both sexes. For this species, our data refute the hypothesis that female ornaments are functionless but are expressed merely as a result of a genetic correlation between the sexes^{20,21}. Our experiments confirm Darwin's¹ suggestion that the evolution of ornaments of monogamous sexually monomorphic animals could be driven by mutual sexual selection. This has profound implications for our understanding of the evolution of sexually monomorphic ornaments in many other bird species. □

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The largest bacterium

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THE large, morphologically peculiar microorganism *Epulopiscium fishelsoni*^{1,2} inhabits the intestinal tract of *Acanthurus nigrofasciatus*, a brown surgeonfish (family Acanthuridae) from the Red Sea. Similar microorganisms have been found in surgeonfish species from the Great Barrier Reef³. As these microorganisms have only been seen in surgeonfish and no free-living forms have been found, they are considered to be specific symbionts of surgeonfish, although the nature of the symbiosis is unclear¹⁻⁴. Initial reports considered them to be eukaryotic protists, based primarily on their size¹⁻⁴, with individuals being larger than 600 µm by 80 µm. But their cellular morphology in the electron microscope is more like that of bacterial than eukaryotic cells^{1,2,5}. To resolve the nature of these symbionts, we have isolated the genes encoding the small subunit ribosomal RNA from two morphotypes and used them in a phylogenetic analysis. *In situ* hybridization with oligonucleotide probes based on the cloned rRNA sequences confirmed the source of the rRNA genes. Our results identify the symbionts as members

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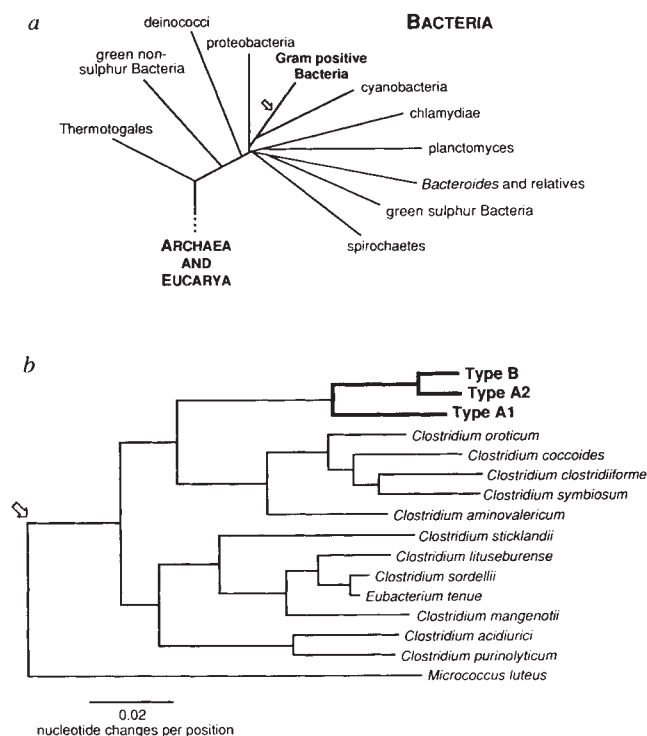
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FIG. 1 Phylogenetic trees relating the surgeonfish symbionts to other bacteria. **a**, Phylogenetic domain of bacteria¹². The tree is generated by least-squares analysis of a distance matrix of small subunit rRNA sequences from representatives of each major bacterial subdivision⁹. The arrow shows the approximate branch point of the symbiont-clostridia cluster, corresponding to the arrow in **b**. **b**, The three surgeonfish symbiont sequences with their closest known relatives based on 16S rRNA sequence comparisons. Type A1 was the predominant sequence type retrieved from the cloned PCR products generated from type A lysates. Type A2 was less abundant in these samples. The type B sequence represents the only category obtained from type B lysates. GenBank accession numbers for type A1, A2 and B are M99572, M99573 and M99574, respectively.

METHODS. For each lysate, about 1,000 cells were washed and disrupted by adding KOH to 0.2M and heating to 70 °C for 15–30 min. Cell lysis was monitored with a dissecting microscope. An equivalent amount of 1M HCl mixed 2:1 with 1M Tris-Cl (pH 8.3) was added to the tube contents, when at least 50% of the cells were lysed. The rRNA genes were amplified by PCR⁶. Symbiont lysate (1 µl) was added to the amplification reaction mixture, which contained 30 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 0.05% Nonidet P-40, 0.2 µg of each primer, 1.25 mM each dATP, dCTP, dGTP and dTTP and 1 unit of *Thermus aquaticus* DNA polymerase in a total volume of 100 µl. The mixture was overlaid with mineral oil and incubated as follows: 1.5 min at 92 °C, 1.5 min at 37 °C, 2 min at 72 °C, for 30 rounds of amplification. PCR products were cloned as *NotI/PstI* fragments¹⁸ in pBlue-script KS⁺ and sequenced using the dideoxy chain-termination method¹⁹.

of the low-(G+C) Gram-positive group of bacteria. They are therefore the largest bacteria to be described so far.

As these symbionts cannot be maintained in culture, a scheme was devised to obtain rRNA gene sequences which circumvents the need for cultivation. Type A and type B (ref. 3) symbionts were collected by micromanipulation from gut contents of *A. nigrofusus* and *Naso tuberosus*, respectively, which were collected on reefs around Lizard Island, Australia (14°40' S, 145°28' E). Individual cells from ethanol-fixed gut contents were transferred five times through a sterile wash solution and disrupted using an alkaline lysis procedure (described in Fig. 1 legend). Lysates were used without further purification as the source of template in a polymerase chain reaction (PCR) to amplify small subunit rRNA genes⁶. Universal primers with polylinker tails, 515FPL (GCGGATCCTCTAGACTGCAGTGCCAGCAGCCGCGGT-AA) and 1492RPL (GGCTCGAGCGGCCGCCCCGGGTAA-



CCTTGTTACGACTT), complementary to regions of rDNA that are conserved among all known organisms, were used in the initial amplifications. When the cloned rDNAs were identified as bacterial in type, a different set of primers, the bacteria-specific primer 8FPL (GCGGATCCGCGGCCGCTGCA-GAGTTTGATCCTGGCTCAG) and 1492RPL, were used to amplify a larger region of the rRNA gene. The PCR products from five separate amplifications were cloned, and a total of 85 clones were partially sequenced and categorized.

Two slightly different rDNA sequences were consistently retrieved from the type A lysates, and only a single sequence was obtained from type B lysates. The heterogeneity of the type A rDNA sequences indicates that this group consists of at least two distinct but closely related strains. This is consistent with the morphological heterogeneity seen in the type A-containing samples. A representative of each of the three classes of clones

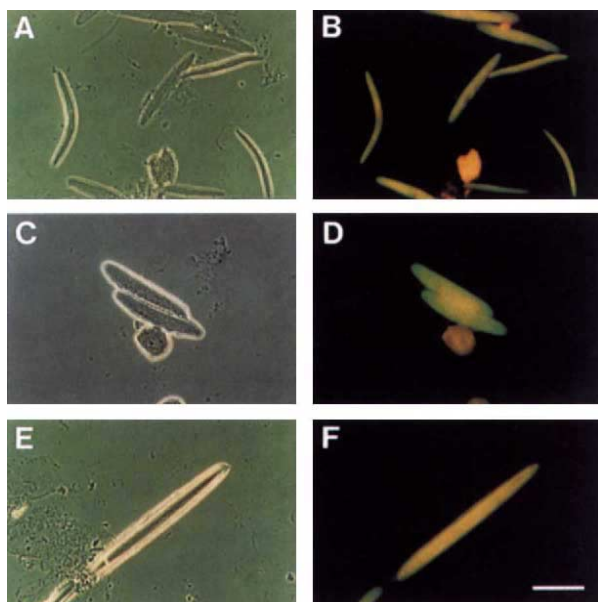


FIG. 2 *In situ* hybridization of gut contents with symbiont-specific, fluorescein labelled oligonucleotides. The oligonucleotides used as symbiont-specific probes, 822R (CCCGTAAGSCCGACACCTAGTATT) and 1423R (TTGCGGTAG-GTCACTGACGTTGGGCCCT), were used for *in situ* hybridization as described¹¹. Panels show pairs of micrographs, phase contrast (left) and epifluorescence (right), of the same field of surgeonfish gut contents. **A** and **B**, Type A-containing samples hybridized with oligonucleotide 1423R. The slight morphological heterogeneity of these cells can be seen, and may correspond with the two types of rRNAs retrieved from this sample. The amber patches in this field are due to the autofluorescence of photosynthetic pigments of algae ingested by the surgeonfish. **C** and **D**, Type B-containing sample hybridized with oligonucleotide 822R. The yellow cell in this field is the ciliate *Balantidium* which also inhabits the intestinal tract of *N. tuberosus* and has a yellowish autofluorescence at this wavelength. **E** and **F**, *Epulopiscium fishelsoni*, symbionts taken from Red Sea surgeonfish, probed with oligonucleotide 1423R. Both probes, 822R and 1423R, hybridize equally well to any of the *Epulopiscium*-like surgeonfish symbionts. The intense, uniform signal is indicative of the large number of ribosomes contained in these cells. All photographs were taken at the same magnification. Scale bar in **F** represents 100 µm.

was sequenced in its entirety. The inferred secondary structures of the symbiont rRNAs show no idiosyncrasies in their expected structural elements or conserved features and therefore there is no obvious evidence for errors introduced during the amplification process. These sequences were aligned with rRNA sequences obtained through the Ribosomal Database Project⁷. A distance matrix analysis⁸ of the sequences grouped the cloned rRNA genes among those from the low-(G+C) Gram-positive bacteria⁹, although G+C content is not particularly low (50–51%). This affiliation was supported by the presence in the rRNAs of 'signature' sequences and structural features specifically diagnostic of that phylogenetic group⁹. A bootstrap analysis¹⁰ confirmed this relationship in 100% of 50 trees generated. The three cloned rRNA genes comprise a coherent phylogenetic group among sequences from other low-(G+C) Gram-positive organisms (Fig. 1).

A critical step in this analysis was to prove that the putative symbiont rRNA clones were derived from the symbionts and not other intestinal flora or contaminants. Two regions of the rRNA genes were identified that are highly conserved among the three clones but not in other known bacterial 16S rRNAs. Fluorescein-labelled oligonucleotides complementary to these regions of the putative symbiont rRNAs were synthesized and used for *in situ* hybridization¹¹ with the contents of surgeonfish gut. Each of these symbiont-specific probes hybridized to the type A and type B surgeonfish symbionts, as well as to *Epulopiscium fishelsoni* taken from Red Sea surgeonfish (Fig. 2). These probes did not hybridize to other intestinal flora or to control slides of *Escherichia coli*, *Bacillus megaterium*, *Saccharomyces cerevisiae* or *Paramecium tetraurelia*. The symbionts also hybridized with a bacteria-specific probe, but they did not hybridize with Eucarya-specific¹² and rRNA-like ('sense' strand) oligonucleotides (data not shown).

The *in situ* hybridization pattern and fluorescence intensity of fluorescein-labelled rRNA probes can indicate aspects of cellular physiology¹¹. Bacteria- or symbiont-specific oligonucleotide hybridization with the surgeonfish symbionts showed a uniform distribution of fluor throughout the cell (Fig. 2), which is consistent with microscopic observations indicative of little subcellular compartmentalization. Transmission electron micrographs of surgeonfish symbionts have revealed potential nucleoids that are much larger than could be contained in typical bacteria. This may indicate an expansion of the surgeonfish symbiont DNA content to allow for the volume of protein synthesis needed to support these large rapidly growing cells². This hypothesis is also in agreement with the intense hybridization signal seen within the symbionts, which is indicative of the large number of ribosomes in these cells.

In terms of cell volume, these surgeonfish symbionts are the largest bacteria yet described. We have observed individual cells that are $>600 \times 80 \mu\text{m}$, more than a million times larger in volume than a typical bacterium such as *E. coli*. Other exceptionally large bacteria rival the length of an average *Epulopiscium* but are much smaller in cell volume. *Spirochaeta plicatilis*, for instance, may reach lengths of $250 \mu\text{m}$ but have a cell diameter of only $0.75 \mu\text{m}$ ¹³. An intestinal inhabitant of tadpoles, *Sporospirillum praeclearum*, can reach a cell diameter of $4 \mu\text{m}$ and lengths of $100 \mu\text{m}$ ¹⁴. Notably large coccoid to ovoid bacteria include *Achromatium oxaliferum* ($100 \times 45 \mu\text{m}$), *Thiovulum majus* (up to $25 \mu\text{m}$ in diameter) and *Prochloron* spp. ($30 \mu\text{m}$ in diameter)¹⁵. Large filamentous microbes include species of *Beggiatoa* such as *B. gigantea*, with individual disc-shaped cells $55 \mu\text{m}$ in diameter and $13 \mu\text{m}$ thick¹⁵, and an unusually large hydrothermal vent isolate with trichomes as thick as $122 \mu\text{m}$ in diameter¹⁶. Even a typical surgeonfish symbiont from the samples analysed ($250 \times 40 \mu\text{m}$) has a substantially greater cell volume than the largest of the cells already mentioned.

It is generally considered that the size of a prokaryotic cell is confined to particular surface-to-volume ratios because the movement of metabolites within the cell is presumed to occur

by diffusion¹⁵. These results, indicating that bacteria can be orders of magnitude larger than previously supposed, challenge that view of the constraints dictating the maximum size of a prokaryotic cell.

These findings also emphasize the vast diversity of microbial life forms that remain to be discovered. Generally, only a small portion (less than 1%) of microorganisms in naturally occurring populations can be cultured in the laboratory¹⁷. Using techniques such as we outline here, information can be obtained about specific uncultivable microorganisms. □

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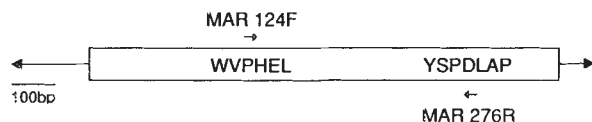
The mariner transposable element is widespread in insects

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THE *mariner* transposable element is a small member of the short inverted terminal repeat class thought to transpose through a DNA intermediate¹. Originally described in *Drosophila mauritiana*², it is now known in several species of the family Drosophilidae^{3,4}, and in a moth *Hyalophora cecropia*⁵. Here I use primers designed to represent regions of amino-acid conservation between the putative transposase genes of the *D. mauritiana* and *H. cecropia* elements to amplify equivalent regions of presumed *mariner* elements from ten other insects representing six additional orders, including the malaria-vector mosquito, *Anopheles gambiae*. Sequences of multiple clones from each species reveal a diverse array of *mariner* elements, with multiple subfamilies in the genomes of some insects, indicating both vertical inheritance and horizontal transfers. An intact open reading frame in at least one clone from each species suggests each may carry functional transposable elements. Therefore the *mariner* element is an excellent candidate for development of genetic transformation systems for non-drosophilid insects, and possibly other arthropods.

Lidholm *et al.*⁵ identified a *mariner* element as an insertion in an intron of the *cecropin A* gene of *H. cecropia*. The genome of this moth contains at least 1,000 copies of *mariner*. The sequenced element is 48% identical to the active *Mos1* element of *D. mauritiana* DNA. Translation of its putative transposase gene required introduction of several deletions, resulting in frameshifts and stop codons, to align with that of the *Mos1*



element, indicating that it is non-functional. The alignment nevertheless revealed 56% amino-acid similarity, with two regions of contiguous six- and seven-amino acid identity ~150 amino acids apart in the middle of the putative transposase. These regions of identity bracket half of the transposase gene, representing a third of this 1,286-base-pair (bp) element.

Degenerate polymerase chain reaction (PCR) primers were designed to represent these two conserved regions (Fig. 1). Amplifications from genomic DNA of *D. mauritiana* and *H. cecropia* yielded a single band of appropriate size (490 bp), indicating that the primers were functioning correctly. Subsequent amplifications yielded a similar PCR product from ten additional species representing six additional orders (Fig. 2a). No amplification was obtained from genomic DNA of 65 other insects, or from 18 vertebrates representing the major classes. Absence of amplification products does not demonstrate absence of *mariner*. Some are likely to be false negatives where single amino-acid replacements in the conserved regions might prevent amplification.

To confirm that these PCR products represent *mariner* elements, they were cloned and at least six clones from each species were sequenced. Most of these sequences recovered one or both of the *D. mauritiana* and *H. cecropia* elements as the best match in Genbank using FASTA⁶. As with the sequence from *H. cecropia*, many required small deletions and insertions for their putative encoded amino-acid sequences to be aligned with the *D. mauritiana* transposase, often resulting in frameshifts and stop codons (Fig. 3).

FIG. 1 Diagram of the *mariner* element showing the contiguous identical amino acids of the *D. mauritiana* and *H. cecropia* elements⁵. The box indicates the open reading frame thought to encode a transposase. The large arrows indicate the terminal inverted repeats of the element and the small arrows the degenerate PCR primers. The scale is for the *Mos1* element. The primers were MAR 124F-5'-TGGGTNCCNCAYGARYT (17-mer, 128-fold degenerate) and MAR 276R-5'-GGNGCNARRTCNGGNSWRTA (20-mer, 4,096-fold degenerate). The number refers to the amino acid in the *Mos1* transposase on which the primer ends and the F (forward) and R (reverse) refer to their orientations with respect to the reading frame (Y=C or T; R=A or G; S=C or G; W=A or T; N=A, C, G, or T).

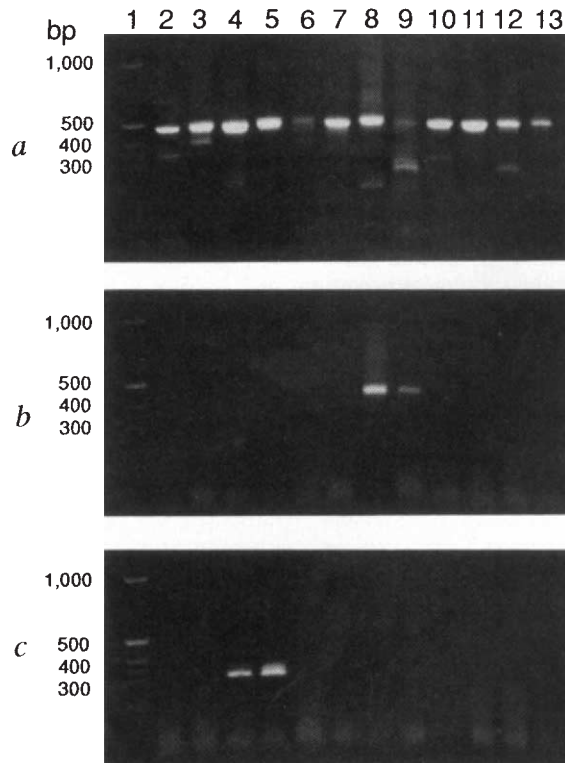
A phylogenetic analysis of these sequences was performed using maximum parsimony as implemented by the computer program PAUP Version 3.0s for the Macintosh⁷, and a representative of the many equally most parsimonious trees is presented in Fig. 4. Several points can be made from this alignment and tree.

First, most of the clones from *D. mauritiana* are very similar to the corresponding region of the *Mos1* element⁸, exhibiting only 0–3 differences at the nucleotide level, within the error rate expected from PCR with *Taq* polymerase for 40 cycles⁹, and the variability already reported for elements from this species^{3,4}. Most of the clones obtained from *H. cecropia* are also similar to the element previously reported⁵. Two control experiments were done to test for any artefacts introduced during PCR. The first was an amplification from the cat flea 10.1 plasmid DNA, from which ten clones were sequenced, yielding an error rate of 4/4,931 bp = 0.081%. The second was an amplification from a cocktail of four plasmid DNAs (clones bean leaf beetle 11.3, deer fly 9.2, honey bee 4.4, and horn fly 3.4), from which twelve clones were sequenced. None showed evidence of chimaeric clones that might result from extended primers annealing to another clone in a later cycle of the PCR, and the error rate was again low (2/5,884 bp = 0.034%). This PCR therefore amplifies faithfully the appropriate segments of *mariner* elements.

Second, the phylogenetic tree indicates that these *mariner* elements can be divided into several major subfamilies. The *D. mauritiana* elements represent one subfamily, whereas the

FIG. 2 a, Amplifications from the twelve insects that were positive for *mariner*. The lanes are: (1) BRL 1 kb ladder (250 ng); (2) fruit fly (*D. mauritiana*, Drosophilidae, Diptera); (3) cecropia moth (*H. cecropia*, Saturniidae, Lepidoptera); (4) earwig (*Forficula auricularia*, Forficulidae, Dermaptera); (5) honey bee (*Apis mellifera*, Apidae, Hymenoptera); (6) bean leaf beetle (*Cerotoma trifurcata*, Chrysomelidae, Coleoptera); (7) silverfish (*Ctenolepisma lineata*, Lepismatidae, Thysanura); (8) horn fly (*Haematobia irritans*, Muscidae, Diptera); (9) mosquito (*Anopheles gambiae*, Culicidae, Diptera); (10) cat flea (*Ctenocephalides felis*, Pulicidae, Siphonaptera); (11) almond moth (*Ephestia cautella*, Pyralidae, Lepidoptera); (12) large milkweed bug (*Oncopeltus fasciatus*, Lygaeidae, Hemiptera); and (13) deer fly (*Chrysops vittatus*, Tabanidae, Diptera). b, Amplifications from the same twelve DNAs using the degenerate primer MAR 124F and a specific primer for the horn fly element, MAR 255R (5'-ATCAAAAGCTGRTATTCATC). Only the horn fly and *A. gambiae* yielded the expected product of ~450 bp. c, Amplifications from the same twelve DNAs using the degenerate primer MAR 276R and a specific primer for the honey bee element, MAR 168F (5'-TTGCCATCGTTCTCAATGAC). Only the honey bee and earwig yielded the expected product of ~300 bp.

METHODS. Genomic DNA was prepared using phenol/chloroform extraction and ethanol precipitation. Single individuals of the cecropia moth, honey bee, large milkweed bug, earwig, and silverfish, and multiple individuals from one population of the others were extracted. All have been confirmed positive for *mariner* by amplifications from separately collected material, as have the amplifications in b and c. The conditions for PCR¹⁹ were Promega's *Taq* polymerase buffer and 2.5 mM MgCl₂, 200 μM dNTPs, 800 nM each primer, 0.25 units Promega *Taq* polymerase, and 2–20 ng genomic DNA in a 25 μl total volume, using cell culture water (Sigma) for dilutions throughout, for 40 cycles (2 min initial denaturation at 95 °C, followed by 40 cycles of 1 min denaturation at 95 °C, 1 min annealing at 50 °C, and 1 min extension at 75 °C, and 5 min at 75 °C to end) in a PTC-100 Thermal Cycler (MJ Research). 10 μl of each reaction were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light.



majority of the *H. cecropia* elements represent a second. A third consists of elements similar to those of the honey bee, and a fourth of elements similar to those of the horn fly. The sequence identity between members of these different subfamilies ranges from 40–56% for DNA and 23–45% for amino acids. The reality of these major subfamilies of *mariner* elements is further supported by the length differences that distinguish three of the subfamilies, features not considered in the phylogenetic analysis. In a subsequent screen of over 400 insects and closely related arthropods (to be published elsewhere), about 15% were positive for *mariner* by this PCR assay. They include the Mediterranean fruit fly (*Ceratitis capitata*), a centipede, and a mite. Sequences from 18 of these support the existence of the four major subfamilies of *mariner* elements, although occasional clones suggest the existence of more subfamilies, as do the earwig 5.1 and 5.10 clones in Fig. 4. Others are probably still undetected by these particular PCR primers.

Third, while all the clones for three species (*D. mauritiana*, milkweed bug, and horn fly) are very similar to each other and form tight clusters in the tree, five species (*H. cecropia*, honey bee, cat flea, almond moth, and *A. gambiae*) have one or two clones that are quite distinct and cluster elsewhere in the tree. The clones from the remaining species (deer fly, bean leaf beetle, earwig, and silverfish) are scattered throughout the tree. These relationships indicate the presence of multiple types of *mariner* elements in some species, sometimes representing different subfamilies. That these relationships do not accord easily with the phylogeny of these insects was demonstrated by constraining the tree to group the sequences according to a generally accepted phylogenetic relationship for the taxa¹⁰, which required 1,111 more steps than the 1,658 in Fig. 4. The major subfamilies of *mariners* might have diverged before the diversification of insects with subsequent loss from some species and/or horizontal transfers to others.

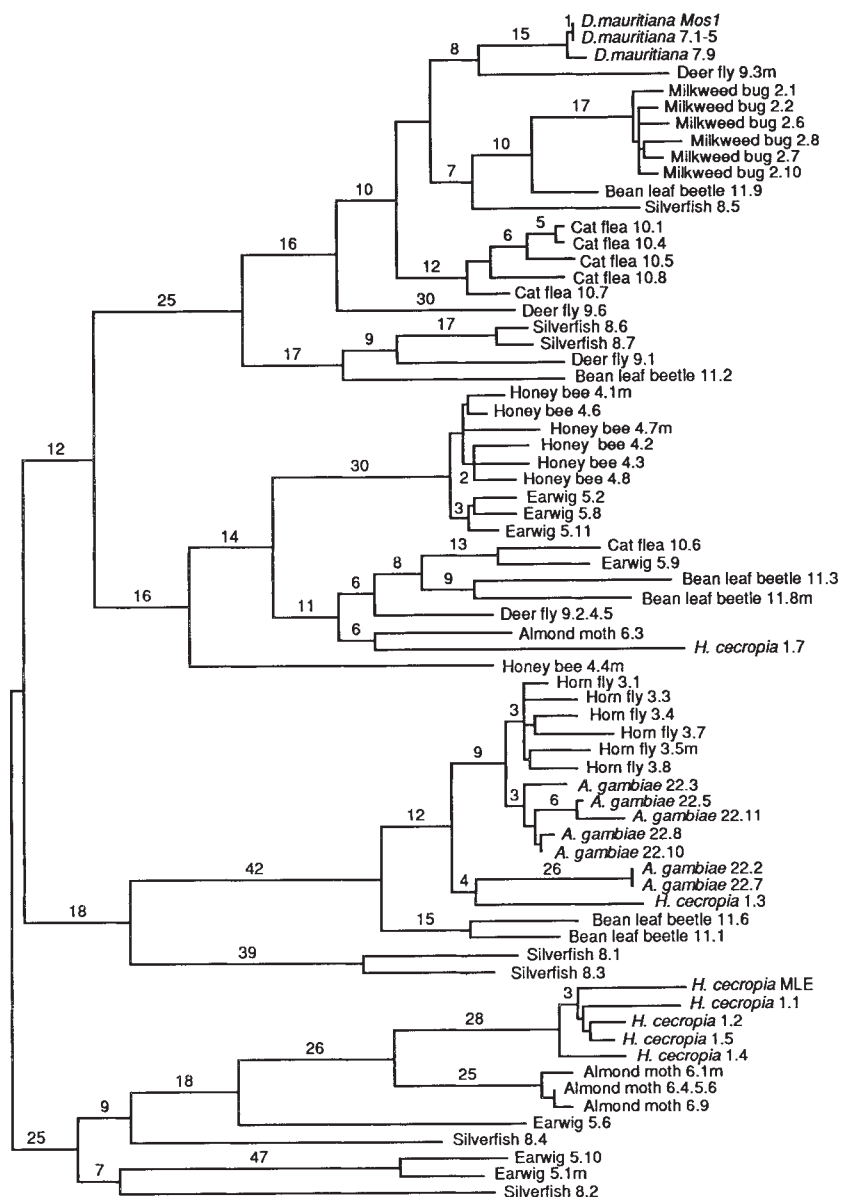
<i>D. mauritiana</i> Mos1	NERQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>D. mauritiana</i> 7.1-5	NERQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>D. mauritiana</i> 7.9	NERQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Deer fly 9.3m	TERQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Milkweed bug 2.1	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Milkweed bug 2.2	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Milkweed bug 2.6	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Milkweed bug 2.8	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Milkweed bug 2.10	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Milkweed bug 2.7	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Bean leaf beetle 11.9	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Silverfish 8.5	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Cat flea 10.1	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Cat flea 10.4	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Cat flea 10.5	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Cat flea 10.7	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Cat flea 10.8	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Deer fly 9.6	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Silverfish 8.6	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Silverfish 8.7	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Deer fly 9.1	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Bean leaf beetle 11.2	DEQMERKNTCEILSSYKRRKSS---FLHRIVTGDCKWIFFVNPQRKSS---YVDPGQATSTARPNGFGKTMLCVMDQSGVYYELLPKGETVNTARVYQOGLINLNRALQRRKPEYQKRQHRVIFLHDNPASHTARAVRDTLETL-NMEVLP HAA
Honey bee 4.1m	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Honey bee 4.2	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Honey bee 4.3	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Honey bee 4.6	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Honey bee 4.7m	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Honey bee 4.8	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Earwig 5.2	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Earwig 5.11	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Earwig 5.10	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Earwig 5.9	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Bean leaf beetle 11.3	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Bean leaf beetle 11.8m	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Deer fly 9.2,4,5	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Almond moth 6.3	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>H. cecropia</i> 1.7	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Honey bee 4.4m	KEKHLTORINSCDLLKRRKNDP---FLKRLITGDCKWVYNNIKRRSS---WSPRESAQTSTAKGIRKRVKLLSVMDQYGVYELLPN-RTINSVYIQLKLNANAEKREELTNRG-VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Horn fly 3.1	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Horn fly 3.3	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Horn fly 3.4	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Horn fly 3.5m	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Horn fly 3.7	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Horn fly 3.8	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.3	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.5	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.8	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.10	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.11	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.2	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.7	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>A. gambiae</i> 22.2	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Bean leaf beetle 11.6	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Bean leaf beetle 11.1	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Silverfish 8.1	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
Silverfish 8.3	TDQKQORVDSERQGLTRNTPFRRYVMTGTHLHPTESMRQSAWATGEPSPKRGKTQSACKVMSVSDAHGIFIDYLEKCKTINSDYVALLERLKVIAIAKRRHMKKK--VIFHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>H. cecropia</i> MLE	SESNLQTRVDCCTILMNNHNEG---KMYDNKRGSLG---WLNQDPAKSCPRKRLITQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>H. cecropia</i> 1.1	SESNLQTRVDCCTILMNNHNEG---KMYDNKRGSLG---WLNQDPAKSCPRKRLITQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>H. cecropia</i> 1.2	SESNLQTRVDCCTILMNNHNEG---KMYDNKRGSLG---WLNQDPAKSCPRKRLITQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>H. cecropia</i> 1.4	SESNLQTRVDCCTILMNNHNEG---KMYDNKRGSLG---WLNQDPAKSCPRKRLITQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
<i>H. cecropia</i> 1.5	SESNLQTRVDCCTILMNNHNEG---KMYDNKRGSLG---WLNQDPAKSCPRKRLITQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Almond moth 6.1m	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Almond moth 6.4,5,6	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Almond moth 6.9	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Earwig 5.6	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Silverfish 8.4	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Earwig 5.10	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Earwig 5.1m	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Silverfish 8.2	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA
Consensus	NDQREVRITCTIALLNRRHNEG---LNRITVDCCKWILFONRRKSSA---WLDGSPAPKQKRRKLTQKLLVSVMTSAGVHVSLKSGQITADYIQLQGLQAMKEALAAKRPVLRNRS-SLLHDNPASHTARAVRDTLETL-NMEVLP HAA

FIG. 3 Alignment of translations of at least six clones of *mariner* PCR fragments from each of the twelve species. The sequences are organized according to their phylogenetic relationships, and the main subfamilies are separated for clarity. The symbol * indicates a frameshift to maintain an aligned reading frame, and an asterisk indicates a stop codon in the aligned reading frame. Clones indicated by an 'm' required removal of 1–9 bp for alignment (which were treated as frameshifts); a period indicates lack of an open reading frame. The consensus sequence indicates in capital letters amino acids shared by most clones in each major subfamily, and a small letter those shared by three subfamilies. Single representatives are shown for multiple clones that were identical in amino-acid translation despite underlying DNA sequence differences, for example, *D. mauritiana* 7.1-5. The *D. mauritiana* Mos1 and *H. cecropia* MLE sequences are the corresponding

regions from references 8 and 5, and are amino acids 125–275 of the Mos1 element.

METHODS. PCR products were separated on a 1.2% NuSieve (FMC) agarose gels, purified by the freeze-fracture method²⁰, and cloned into ddT-tailed pCDNA1 (in Vitrogen) plasmid vector²¹. Cloning and plasmid minipreps²² were by standard methods²³. The inserts were sequenced enzymatically²⁴ using Sequenase v2.0 (USB) on both strands for most of their length. Compressions in regions near the primers that were only sequenced on one strand were resolved with the use of dITP. Sequences were aligned manually using the FASTA alignments from Genbank as a guide, with confirmation using Clustal V (ref. 25). They were translated using DNA Strider 1.1, with judicious use of frameshifts to maintain an aligned reading frame. The translations were again aligned using Clustal V, with final manual adjustment.

FIG. 4 An arbitrary representative of the many most parsimonious trees of the *mariner* phylogeny. Gaps and frameshifts were treated as missing characters. The Heuristic option of PAUP⁷ was used, with random addition of sequences and TBR branch swapping. Ten iterations were done, each terminated after accumulating over 1,000 equally parsimonious trees of 1,658 steps, with a consistency index of 0.611. The tree was rooted at the midpoint, because no outgroup is available for comparison. Branch lengths (in number of amino-acid changes) are indicated for those nodes that were present in 100% of the trees from the final run, according to the semi-strict consensus option of PAUP. Trees derived using the PROTPARS option, or from the DNA sequences using only first and second codon positions, have the same features of topology relevant to the arguments presented here, any differences being in the poorly resolved relationships of the major subfamilies to each other and the unusual clones such as the silverfish 8.2, 8.4 and earwig 5.1, 5.10 clones.



Finally, the occurrence of recent horizontal transfers is strongly indicated by the relationships of the *mariners* from some species. The majority clones from the horn fly and *A. gambiae* are closely related (91–96% DNA and 80–90% amino acid identity), as are the majority clones from the honey bee and the earwig (94–96% DNA and 86–91% amino acid identity). The presence of the horn fly type element in *A. gambiae* and the honey bee type in the earwig was confirmed using PCR primers specific to these elements (Fig. 2b, c). The two flies represent the suborders Cyclorrhapha and Nematocera respectively, which last shared a common ancestor at least 200 Myr ago¹¹. The honey bee and earwig represent the principal insect divisions Endopterygota and Exopterygota respectively, which last shared a common ancestor at least 265 Myr ago¹². It is unlikely that a transposon would be conserved at 91–96% of nucleotide positions for these durations. For comparison, the P element from *Scaptomyza pallida*, which is in the family Drosophilidae, is only 78% identical to the *Drosophila melanogaster* P element¹³. Nor is it likely that these elements could have converged in sequence to this extent. Indeed, most of these nucleotide changes are non-synonymous, indicating relatively unconstrained evolution of these elements. Their pres-

ence in such distantly related insects therefore most probably resulted from horizontal transfer. On the other hand the *mariners* from the two moths are relatively closely related (64–67% DNA and 57–65% amino acid identity) indicating possible vertical inheritance. Similar patterns of vertical inheritance and horizontal transfer have been reported for *mariner*⁴ and other transposons¹⁴ in various Drosophilids, as well as for endosymbiotic bacteria of insects¹⁵. Whatever the mechanism¹⁶, the *mariner* phylogeny implies that horizontal transfers have occurred relatively frequently and over great taxonomic distances. Horizontal transfers might even be integral to the persistence of these 'selfish' genetic elements¹⁷.

The widespread but apparently sporadic distribution of *mariner* elements suggests that they might be developed as genetic transformation systems for non-drosophilid insects and other arthropods. The *Mos1* element from *D. mauritiana* is capable of transposition into the genome of *D. melanogaster*¹⁸. It is possible that it will function in all arthropods. Alternatively, elements from close relatives of the target species might be used. For each species described here, at least one clone was recovered with no major deletions, frameshifts or stop codons, suggesting that each species might be found to contain elements with intact

open reading frames. Such elements might be fully functional and useful as genetic transformation vectors. □

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Disruption of the murine IL-4 gene blocks Th2 cytokine responses

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MURINE T-helper clones are classified into two distinct subsets (Th1 and Th2) on the basis of their patterns of lymphokine secretion. Th1 clones secrete interleukin-2 (IL-2), tumour necrosis factor- β (TNF- β) and interferon- γ (IFN- γ), whereas Th2 clones secrete IL-4, IL-5 and IL-10 (ref. 1). These subsets are reciprocally regulated by IL-4, IL-10 and IFN- γ and differentially promote antibody or delayed-type hypersensitivity responses^{2,3}. To evaluate whether IL-4 is required for mounting Th2 responses, we generated IL-4-mutant mice (IL-4^{-/-})^{4,5} and assessed the cytokine secretion pattern of T cells both from naive and *Nippostrongylus brasiliensis* infected mice. CD4⁺ T cells from naive IL-4^{-/-} mice failed to produce Th2-derived cytokines after *in vitro* stimulation. The levels of Th2 cytokines IL-5, IL-9 and IL-10 from CD4⁺ T cells obtained after nematode infection were significantly reduced. The reduced IL-5 production in IL-4^{-/-} mice correlated with reduced helminth-induced eosinophilia, which has been shown to be dependent on IL-5 *in vivo*⁶. We conclude that IL-4 is required for the generation of the Th2-derived cytokines and that immune responses dependent on these cytokines are impaired.

The strategy for disrupting the IL-4 gene is detailed in Fig.

1. F₁ mice (129Sv×C57BL/6) heterozygous for the mutation (IL-4^{+/-}) derived from one embryonic stem (ES) cell clone were interbred to obtain mice homozygous for the disrupted IL-4 gene (IL-4^{-/-}). Loss of the wild-type allele was confirmed by Southern blotting (Fig. 1d). Interbreeding of (129Sv×C57BL/6) F₂ IL-4^{-/-} mice gave rise to a stock of mice that were used for experiments. They were compared with sex- and age-matched mice of IL-4-wild-type (IL-4^{+/+}) F₂ breedings.

In vitro, IL-4 promotes the development of Th2 cells⁷⁻⁹. We tested the ability of CD4⁺ T cells from naive IL-4^{-/-} and IL-4^{+/+} mice to produce the Th2 cytokines IL-3, IL-4, IL-5 and IL-10. CD4⁺ T cells were stimulated as described in Fig. 2a. Whereas cells from control mice stimulated with anti-CD3 produced IL-4, IL-5 and IL-10, these cytokines were undetectable in the culture medium of IL-4^{-/-} cells. IL-3, a cytokine produced by Th1 as

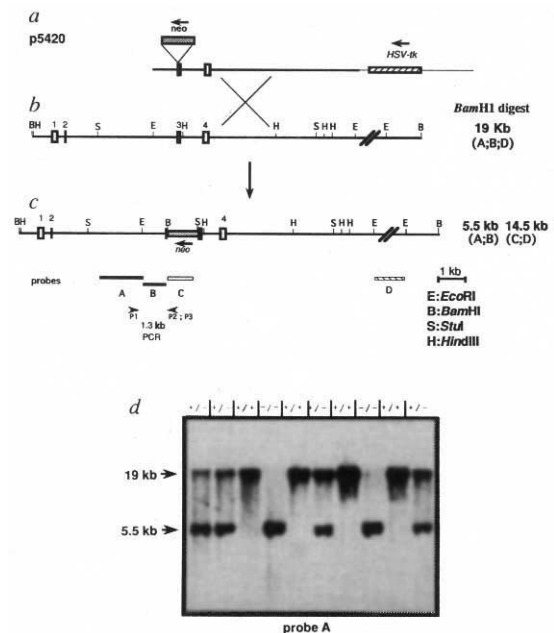


FIG. 1 Disruption of the IL-4 gene. *a*, Schematic structure of the targeting vector p5420. The neomycin (*neo*) gene cassette contains the 1.2-kb (*XhoI/SalI*) fragment of the plasmid pmcplA (Stratagene) and was inserted into a *XhoI* site created at a previous *SacI* site in exon 3 of the 7.5-kb genomic *EcoRI* fragment of IL-4 (arrow indicates the transcriptional orientation of the *neo* gene). The HSV-tk cassette²¹ (*HindIII/XhoI* fragment) was blunted and inserted at the blunted *AflIII* site from pTZ19R (Pharmacia). The vector was linearized with *SalI* before electroporation into D3 (ref. 22) embryonic stem cells. Positive and negative selection was used to enrich for homologous recombinants²¹, which were identified by analysis by polymerase chain reaction (PCR) analysis. *b*, Genomic structure of the murine IL-4 gene. Exons are represented by boxes. *c*, The predicted structure of the targeted allele following homologous recombination with p5420. The configuration of the targeted IL-4 allele was checked by digesting genomic DNA from ES clones with *BamHI*, *HindIII* and *StuI* (not shown). All targeted ES clones were tested for the integration of only one *neo* gene (panel *c*, probe C) and for the absence of genomic rearrangements downstream of the mutated exon (panel *c*, probe D). The expected sizes of restriction fragments that would be detected by the indicated probes after *BamHI* digestion of wild-type and mutated alleles are shown. Primers specific for the *neo* gene (P2: GCGCATCGCCTTCTATCGCC) and for the IL-4 gene (P1: CTAGGCTCTCACATGCTAG) were used for DNA amplification of specifically recombined clones. *d*, Southern blot analysis of the IL-4 locus with tail DNA of progeny derived from intercrossing of heterozygous littermates. The genotype is indicated above each lane. IL-4^{+/+}, wild type; IL-4^{+/-}, heterozygous mutant; IL-4^{-/-}, homozygous mutant. 15 μ g DNA were digested with *BamHI*, run on a 0.8% agarose gel and blot-hybridized with probe A (*d*) by standard procedures. The functional disruption of the IL-4 gene has been confirmed by both the absence of IL-4 mRNA as measured by reverse transcriptase-PCR and bioactivity as determined by proliferation of the IL-4-sensitive cell line 4S (ref. 23) using supernatants from anti-CD3 plus IL-2-stimulated IL-4^{-/-} CD4⁺ T cells (not shown).

well as Th2 clones, was reduced by more than 70% of control values. IFN- γ secretion, however, is comparably stimulated from both mutant and wild-type cells. The addition of anti-IL-4 monoclonal antibody to control cultures mimicked the mutant phenotype, whereas the addition of IL-4 as cofactor during the prestimulation culture induced the production of IL-5 and IL-10 by IL-4 $^{-/-}$ CD4 $^{+}$ T cells (Fig. 2a). IL-4 is therefore a necessary factor for the generation of the Th2-cell cytokine profile *in vitro*. This effect may be due to a selective proliferation of Th2 cells or to the promotion of Th2 differentiation.

To determine whether IL-4 is necessary for the generation of the Th2 phenotype *in vivo*, we analysed the cytokine profiles of lymph node cells from mice infected with *N. brasiliensis* (*Nb*). This nematode infection has several effects on the immune system which are consistent with a selective expansion, activation or differentiation of Th2 cells¹⁰. Mesenteric lymph node cells from *Nb*-infected IL-4 $^{-/-}$ and IL-4 $^{+/+}$ mice were depleted of B cells and CD8 $^{+}$ or CD4 $^{+}$ T cells, stimulated for 24 hours with anti-CD3 antibody and IL-2 and assayed for lymphokine production. As shown in Fig. 2b, the cytokines IL-3, IL-5, IL-10 and also IL-9 from IL-4 $^{+/+}$ T cells correlated with the IL-4 (Th2) response as they were absent in pre-immune supernatants but were augmented 15- to 300-fold in supernatants from CD4 $^{+}$ T cells obtained from IL-4 $^{+/+}$ mice at day 7 post-infection. In contrast, supernatants of CD4 $^{+}$ T cells from IL-4 $^{-/-}$ mice had reduced levels of Th2 cytokines. Interestingly, we found that

some IL-5 was also produced from CD8 $^{+}$ T cells both from IL-4 $^{+/+}$ as well as IL-4 $^{-/-}$ mice after *Nb* infection (results not shown). As shown previously¹¹, IL-5 is also produced by non-CD4 $^{+}$ cells in mice injected with anti-IgD antibody.

To our knowledge, our results provide the first evidence for the IL-4-dependency of Th2 cytokine response *in vivo*. Residual Th2 cytokine levels in IL-4 $^{-/-}$ mice may come from a minor Th subset distinct from the classical Th2 subset, which appears to be IL-4-independent. Alternatively, virgin Th cells (Thp) or short-term stimulated Th cells (Th0) can be activated for Th2 cytokine secretion in an IL-4-independent manner, whereas chronically stimulated Th cells are IL-4-dependent.

In vitro, Th1 and Th2 subsets are cross-regulated by IL-4, IL-10 and IFN- γ (refs 3, 12). Indeed, CD4 $^{+}$ T cells from infected IL-4 $^{-/-}$ mice produced higher levels of IFN- γ than controls (Fig. 2b), possibly reflecting the missing negative feedback from IL-4 or IL-10. But the primary source of IFN- γ production was CD8 $^{+}$ T cells, with levels of secretion comparable in wild-type and IL-4 $^{-/-}$ mice (Fig. 2c). Surprisingly, in one of two experiments CD4 $^{+}$ T cells from uninfected controls had detectable IFN- γ levels. This may be accounted for by contaminating CD8 $^{+}$ T cells or infection with natural antigens.

To have an independent *in vivo* measurement for T cell function, delayed-type hypersensitivity DTH reactions were tested by infection with lymphocytic choriomeningitis virus (LCMV). LCMV-induced DTH responses are mediated initially

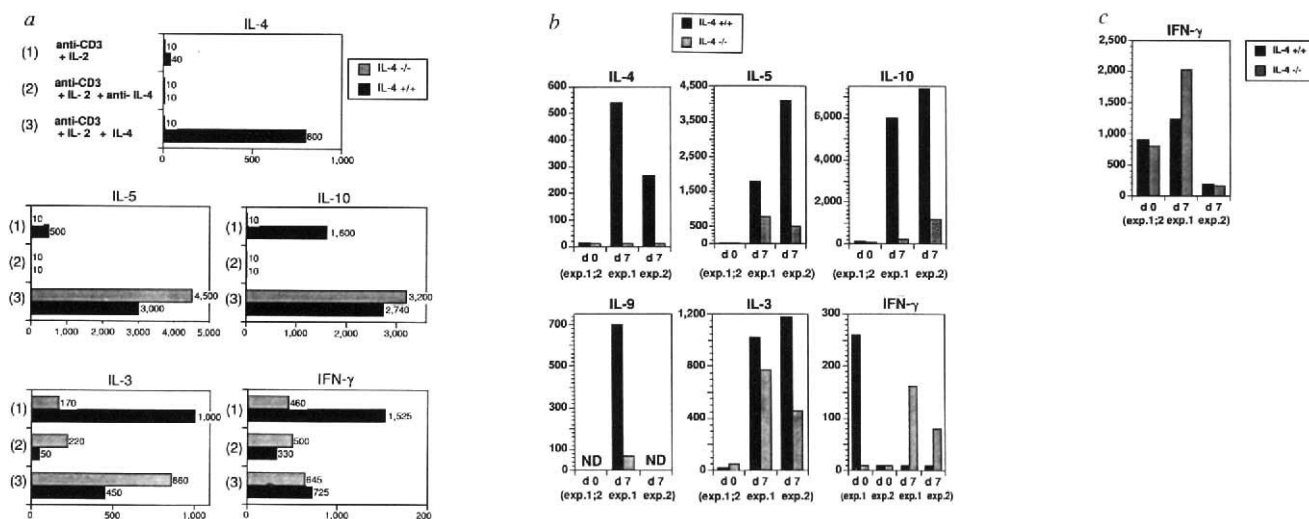


FIG. 2 Cytokine secretion after polyclonal T-cell stimulation. *a*, *In vitro* induction of Th2 cells. CD8 $^{+}$ - and Ig $^{+}$ -depleted cells from the mesenteric lymph nodes of naive IL-4 $^{+/+}$ and IL-4 $^{-/-}$ were stimulated under the conditions indicated for 5 d, washed, and restimulated with anti-CD3 and IL-2 for 24 h. Supernatants of the last 24 h of culture were assayed for cytokines IL-3, IL-4, IL-5, IL-10 and IFN- γ by ELISA and standardized to commercial cytokines (Genzyme). Values are given as units per 2×10^5 cells. The threshold for detection of IL-10 was 20 U; all others were 10 U. *b*, Cytokine secretion after *Nb* infection. CD8 $^{+}$ - and Ig $^{+}$ -depleted cells from the mesenteric lymph nodes (MLN) of IL-4 $^{-/-}$ and IL-4 $^{+/+}$ pre-(d0) and post-(d7)-*Nb* infection were stimulated for 24 h on an anti-CD3-coated plate in the presence of IL-2 (50 U ml $^{-1}$). Supernatants were analysed for cytokines IL-3, IL-4, IL-5, IL-10 and IFN- γ by ELISA. IL-9 was measured with the IL-9-dependent T-cell subclone ST2/K9.4a2 (ref. 28). Cytokine production was dependent on anti-CD3 treatment (not shown). Values are given as units per 2×10^5 cells from 2 independent experiments (exp. 1; exp. 2) with MLN pooled from 4 mice (exp. 1) and 5 mice (exp. 2) per group. ND, not determined. *c*, IFN- γ production from CD4 $^{+}$ - and Ig $^{+}$ -depleted MLN cells pre- and post-infection with *Nb*. Cell stimulation and cytokine measurements were done as described in *b*. CD4 $^{+}$ T-cell depletion was achieved with monoclonal antibody GK1.5.

METHODS. *a*, CD4 $^{+}$ T cells were prepared by selective removal of CD8 $^{+}$ T cells and B cells using a combination of rat anti-CD8, 2.43 (ref. 24) and sheep anti-rat-Ig and sheep anti-mouse Ig magnetic beads (Dyna, Norway) followed by two cycles of exposure to a magnetic field. Cell purity was

determined by cytometric analysis (FACScan, Becton Dickinson) using FITC-labelled anti-CD4 monoclonal antibody GK1.5 (ref. 25) and was in the range 93–95% CD4 $^{+}$ cells. CD4 $^{+}$ cells were cultured on anti-CD3 ϵ (20 μ g ml $^{-1}$) monoclonal antibody 2C11 (ref. 26) in 50 μ l bicarbonate-buffered saline, pH 8.6 coated dishes (Falcon) in the presence of (1) IL-2 (50 U ml $^{-1}$; Boehringer Mannheim); (2) IL-2 + anti-IL-4 monoclonal antibody (40 μ g ml $^{-1}$) (ref. 27) or (3) IL-2 + IL-4 (1,000 U ml $^{-1}$). After the 5-d culture period, cells were washed and restimulated for 24 h at a density of 2×10^5 cells per well on an anti-CD3-coated 96-well plate (Costar) in the presence of IL-2 (50 U ml $^{-1}$). Antibodies for measuring IL-3, IL-4 and IL-5 were obtained from Pharmingen; antibodies against IFN- γ , XGM-6 and R46A2 were a gift from F. Finkelman and S. Alkan. Anti-IL-10 monoclonal antibodies SX-1 and SX-2 were a gift from T. Mosmann. All cells were cultured in DMEM containing 10% FCS, 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM) and gentamycin (50 μ g ml $^{-1}$). *b*, Mice were subcutaneously injected with 750 *Nb* L3, prepared as described²⁹. Cell depletion and cytokine measurements were done as described for *a*. For IL-9 determination, 5×10^3 ST2/K9, 4a2 cells (gift from E. Schmitt) were cultured with serial sample dilutions in the presence of 20 μ g ml $^{-1}$ anti-IL2 (S4B6) and 10 μ g ml $^{-1}$ anti-IL-4 (11B11). After 24 h, cells were incubated with 3 H-thymidine (0.1 μ Cl per well) for a further 24 h and DNA synthesis measured. For standardization we used mL-9, derived from a baculovirus supernatant (4×10^5 U ml $^{-1}$); gift from J. Van Snick.

by CD8⁺ T cells followed by CD4⁺ T cells^{13,14}. Injection of replicating LCMV into footpads of both IL-4^{-/-} and control mice induced a CD8⁺ T cell-mediated swelling which peaked on day 8, followed by a CD4⁺ T cell-mediated swelling which peaked on day 11 (manuscript in preparation). These results suggest that IL-4^{-/-} mice are not impaired in DTH reactions but have a selective Th2 defect.

We next measured serum immunoglobulin in naive and immunized mice. Serum immunoglobulin concentrations in non-immunized IL-4^{-/-} mice showed 20-fold decreased IgG1 levels and undetectable IgE (<0.015 µg ml⁻¹). The serum levels of other immunoglobulin isotypes were not significantly altered (Fig. 3a). After anti-IgD treatment (not shown) or infection with *Nb*, both potent inducers of IgG1 and IgE responses¹⁵, IgE levels in IL-4^{-/-} mice remained undetectable, whereas the mice were able to mount an IgG1 response. But IgG1 levels were still 12-fold reduced compared with IL-4^{+/+} mice. Serum levels of IgG2 and IgG3 in *Nb*-infected IL-4^{-/-} mice were slightly increased compared to controls (Fig. 3a). These data confirm previous reports which showed that IgE production is uniquely dependent upon IL-4 (refs 15–17), whereas IgG1 secretion can be induced in the absence of IL-4 (ref. 18, 19). The residual production of IgG1 in IL-4^{-/-} mice is therefore independent of IL-4 and maybe also of other Th2 cytokines.

We further immunized mice with the soluble protein derivative dinitrophenol-ovalbumin (DNP-Ova) DNP-Ova-specific IgG1 antibodies were 10-fold lower, whereas IgG2 and IgG3 isotypes were 100- to 500-fold higher in IL-4^{-/-} mice compared to control levels (not shown), demonstrating the shift in dominance from IgG1 in IL-4^{+/+} mice to IgG2 and IgG3 in IL-4^{-/-} mice, also described in ref. 17. We conclude that genetically induced IL-4-deficiency and impaired Th2 cytokine production do not result in reduced B-cell help, but in an altered isotype pattern.

To assess the physiological effects of reduced IL-5 levels, we quantitated blood eosinophilia in mice after inoculation with *Nb*. During the course of a primary infection, IL-4^{-/-} mice showed a 2–3-fold lower eosinophilia than control mice (Fig. 4a). Upon secondary challenge, IL-4^{-/-} mice had a comparable number of eosinophils up to day 7 and then numbers decreased, whereas numbers in IL-4^{+/+} mice increased (Fig. 4b). Injection of recombinant IL-5 into *Nb*-infected IL-4^{-/-} mice completely

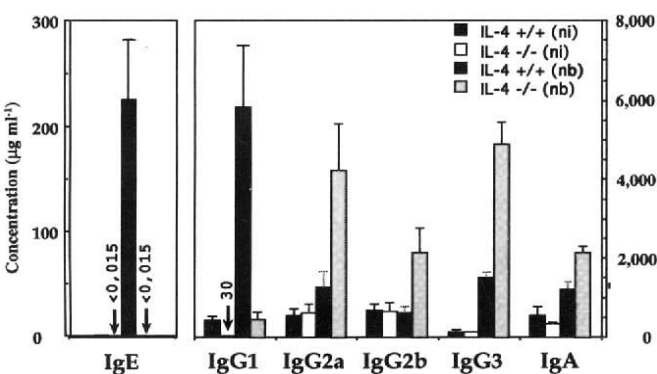


FIG. 3 Serum antibody levels before and after secondary challenge with *Nb*. Eight-week-old mice (4 per group) were subcutaneously injected with 750 *Nb* larvae stage 3 (*Nb* L3) and reinfected 4 weeks later. Serum was taken before (ni, non-immune) and at day 11 post-secondary *Nb* infection (nb). Antibody concentrations were calculated from serially diluted serum samples. Results are given as arithmetic means (+s.e.).

METHODS. For ELISA, Nunc Immuno plates were coated with 5 µg ml⁻¹ goat anti-mouse IgG1, -IgG2a, -IgG2b, -IgG3, -IgM, -IgA (Southern Biotechnology), anti-mouse-IgE monoclonal antibody. Bound immunoglobulin of diluted serum samples was detected with alkaline phosphatase-coupled isotype-specific goat anti-mouse-Ig antibodies (SBA) or with anti-IgE monoclonal antibody, 95.3 (ref. 30). Serum immunoglobulin concentrations were calculated using monoclonal antibodies of the various isotypes (SBA) or IgE monoclonal antibody as standards.

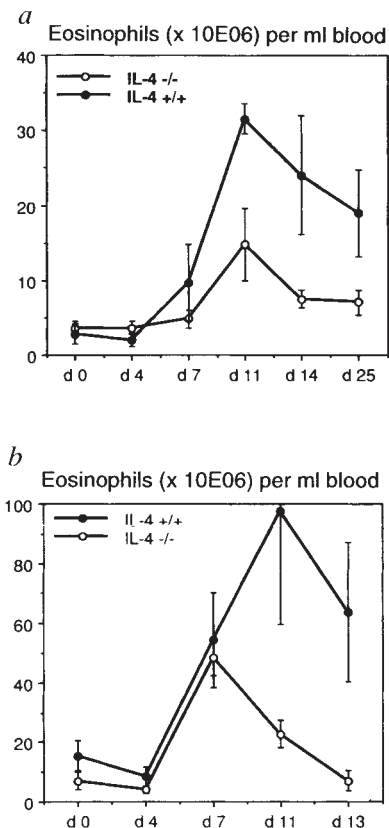


FIG. 4 Blood eosinophilia during the course of a, primary and b secondary infection with *Nb*. 6-week-old mice (4 per group) were infected and reinfected as described above. Peripheral blood was sampled at the indicated days after infection (a) or reinfection (b). Whole blood cells counts were determined in a Sysmex microcell counter (CC170M) and the percentage of eosinophils determined from blood smears stained with Dif Quick (Baxter Dade AG). Data are represented as the mean eosinophil counts per ml blood (+s.e.).

restored the level of eosinophilia (not shown). This confirms that IL-5 is responsible for eosinophilia *in vivo*⁶ and that IL-4 is required for the production of IL-5 from CD4⁺ T cells (Fig. 2b). The presence of eosinophils in IL-4^{-/-} mice is most probably due to residual levels of IL-5 produced from CD4⁺ T cells (Fig. 2b) or from non-CD4⁺ cells^{11,20}.

Taken together, our data support an important role of IL-4 in establishing a functional Th2 immune response. The IL-4-deficient mouse model opens the possibility to study the role and functional contribution of Th2 in allergy and infectious diseases. □

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Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses

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THE discovery of new cytokines normally relies on a prior knowledge of at least one of their biological effects, which is used as a criterion either for the purification of the protein or for the isolation of the complementary DNA by expression cloning. However, the redundancy of cytokine activities complicates the discovery of novel cytokines in this way, and the pleiotropic nature of many cytokines means that the principal activities of a new cytokine may bear little relation to that used for its isolation. We have adopted an alternative approach which relies on differential screening of an organized subtracted cDNA library from activated peripheral blood mononuclear cells, using the inducibility of lymphokine messenger RNAs by anti-CD28 as a primary screening criterion. The ligation of the CD28 antigen on the T lymphocyte by a surface antigen, B7/BB-1, expressed on activated B lymphocytes and monocytes^{1,2} is a key step in the activation of T lymphocytes and the accumulation of lymphokine mRNAs³. Here we report the discovery by molecular cloning of a new interleukin (interleukin-13 or IL-13) expressed in activated human T lymphocytes. Recombinant IL-13 protein inhibits inflammatory cytokine production induced by lipopolysaccharide in human peripheral blood monocytes. Moreover, it synergizes with IL-2 in regulating interferon- γ synthesis in large granular lymphocytes. Recent mapping of the IL-13 gene shows that it is closely linked to the IL-4 gene on chromosome 5q 23–31 (ref. 4). Interleukin-13 may be critical in regulating inflammatory and immune responses.

Systematic sequencing of several hundred cDNAs selected by this differential screening approach has revealed one encoding a novel cytokine (NC30) derived from a 1.4-kilobase (kb) mRNA whose expression is regulated by anti-CD28 (Fig. 1A). The protein encoded by this cDNA was designated interleukin-13 at the 1993 Keystone Cytokine Symposium. The level of induction of IL-13 mRNA with anti-CD28 is less than that observed for interferon (IFN)- γ mRNA, but it is comparable to that observed for granulocyte macrophage colony-stimulating

factor (GM-CSF) mRNA (Fig. 1A). This increase in IL-13 mRNA with anti-CD28 is also seen in highly purified T-lymphocyte preparations (Fig. 1B). The IL-13 cDNA (Fig. 2a) has an open reading frame with two possible methionine initiation codons, the second being present in a nucleotide sequence context closer to the consensus sequence CCA/GCCAUGG for translation initiation⁵. The amino-terminal region of the predicted IL-13 polypeptide is hydrophobic, resembling the signal peptides commonly found in secreted proteins⁶. In the 3' non-coding region of the mRNA there are 4 copies of the (A/T)ATT(A/T) sequence associated with unstable mRNAs, including those of cytokines^{7,8}.

Characterization of genes adjacent to the IL-4 gene⁴ has revealed the partial sequence of a closely linked gene, now identified as the IL-13 gene. The IL-13 protein sequence shows only limited sequence homology with IL-4 (20–25% identity; Fig. 2b). However, the first and last α -helical regions of IL-4 (ref. 9), which are critical for its activity¹⁰, do show homology with IL-13 (Fig. 2b). The IL-13 protein sequence shows ~60% identity with a protein predicted from the mouse P600 cDNA (Fig. 2b), which was identified by differential screening of a concanavalin A-induced mouse C1.Ly-1⁺2⁺/9 lymphocyte cDNA library¹¹. P600 mRNA is expressed by the Th2 mouse lymphocyte subset, which also expresses IL-3, GM-CSF, IL-4

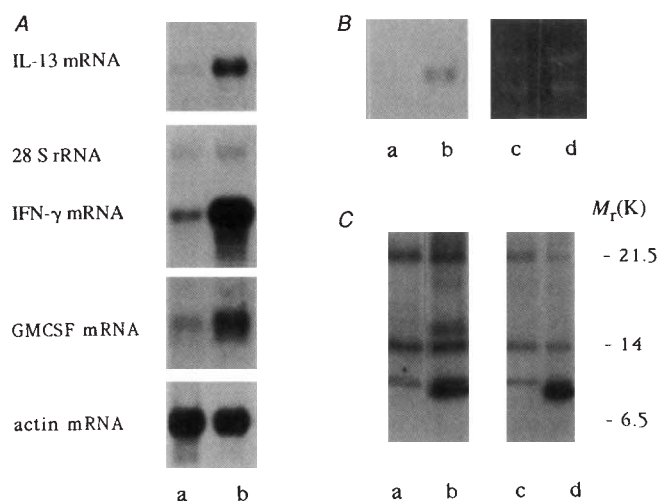


FIG. 1 IL-13 mRNA is inducible in T lymphocytes and encodes a secreted protein. **A**, Induction of IL-13 mRNA in PBMC. Blots of RNAs from non-adherent PBMC, cultured for 5 h with 10 ng ml⁻¹ phorbol myristyl acetate (PMA) (lane a), with 10 ng ml⁻¹ PMA + 1 μ g ml⁻¹ anti-CD28 (248.23.2 anti-CD28 monoclonal antibody from S. Carrel) (lane b) hybridized to IL-13, human interferon- γ , human GMCSF, and mouse β -actin cDNA probes. Details of the pSE1 cloning expression vector and of the construction, organization and screening of the cDNA library are given elsewhere (A.M. *et al.*, manuscript in preparation). **B**, Induction of IL-13 mRNA in purified T lymphocytes. Blots of RNA from purified T lymphocytes cultured for 5 h with 3 ng ml⁻¹ PMA (lanes a, c), or 3 ng ml⁻¹ PMA + 1 μ g ml⁻¹ anti-CD28 (lanes b, d) hybridized to IL-13 cDNA (lanes a, b). We confirmed equal amounts of RNA by ethidium bromide staining of 18S and 28S rRNAs (lanes c, d). The cell population highly enriched in T lymphocytes (85% CD4⁺, CD8⁺, CD3⁺ cells; 10% CD4⁺, CD8⁺, CD3⁺ cells; <1% monocytes) was prepared by negative selection using a cocktail of monoclonal antibodies directed against CD8, CD11b, CD14 and CD24 cell-surface antigens and magnetic beads (Dynal) coated with anti-mouse IgG2. **C**, Secretion of IL-13 proteins by transfected COS cells. Secreted proteins from COS cells transfected with the cloning-expression vector pSE1 (lanes a, c), or with pSE1-IL13 (lanes b, d), and labelled in the presence (lanes c, d) or absence (lanes a, b) of tunicamycin, were analysed on a 15% polyacrylamide-SDS gel. IL-13 proteins are indicated by arrows and the migration of molecular weight markers is shown on the right. COS cells (10⁶) were transfected with 6 μ g plasmid DNA, using DEAE-dextran and chloroquine followed by a dimethylsulphoxide shock²³. After 40 h, cells were labelled for 6 h using 200 μ Ci ³⁵S-methionine with or without 10 μ g ml⁻¹ tunicamycin; the culture medium was collected for analysis.

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and IL-10, but not by the Th1 subset, which expresses IL-3, GM-CSF, IL-2 and IFN- γ ¹².

The function of the protein encoded by the P600 mRNA has not been established, but it has been suggested¹¹ that it may be membrane-anchored rather than secreted. When, however, IL-13 cDNA is expressed in transfected COS cells, it encodes secreted polypeptides with apparent M_s of 9,000 and 17,000 (Fig. 1C). The 17K species is not detected when the cells are labelled in the presence of tunicamycin, suggesting that it represents an N-glycosylated form of IL-13 (Fig. 1C). The IL-13 protein



FIG. 2 Nucleotide sequence of the IL-13 cDNA and comparison of the IL-13 protein with P600 and IL-4. **a**, Nucleotide sequence of the IL-13 cDNA. The cDNA sequence has been submitted to the EMBL Data Library (accession number X69079). The probable methionine initiation codon (see text) is used for numbering the amino acids, and amino acids in the open reading frame preceding this methionine are indicated in italics. The glycine residue at position 21 at the amino terminus of the protein secreted by transfected COS and Chinese hamster ovary cells is indicated by an arrow. The recombinant yeast protein begins two amino acids upstream at Ser 19. Potential N-glycosylation sites (Asn-X-Ser/Thr)²⁴ are indicated by asterisks, and (A/T)ATTA(A/T) motifs^{7,8} are underlined. At amino acid 61, we have isolated cDNAs encoding (cDNA I) Asp (GAC) and (cDNA II) Gly (GGC). The recombinant proteins are encoded by cDNA I. Form II is the more prevalent IL-13 mRNA, however, and form I may represent either a rare allelic variant, or an enzymatic error during construction of the cDNA library. **b**, Sequence alignment of proteins encoded by IL-13, mouse P600 and human IL-4 cDNAs. Mouse P600 (ref. 11), IL-13 and human IL-4 protein sequences are aligned as described (ref. 25). α -Helical regions in IL-4 (ref. 9) are underlined.

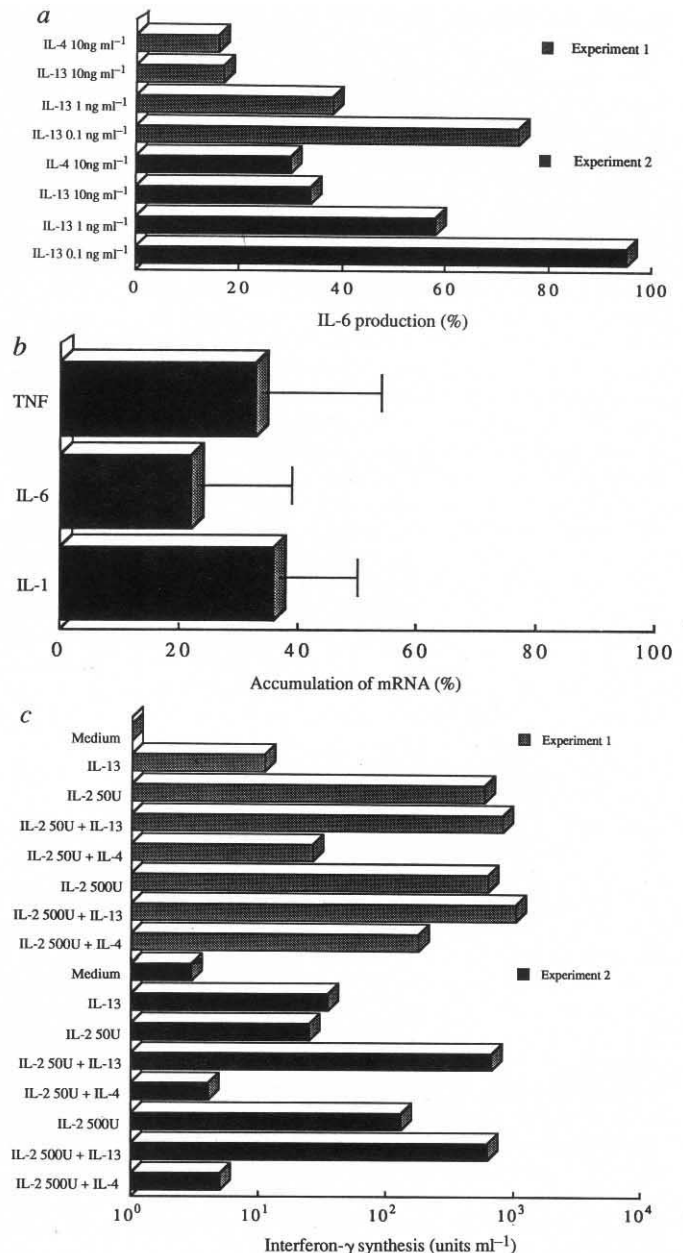


FIG. 3 Biological activities of IL-13. **a**, Inhibition of IL-6 secretion in PBMC. IL-6 production in PBMC incubated for 24 h with 1 μ g ml⁻¹ LPS (*E. coli*; Sigma) and the indicated concentrations of IL-13 or IL-4, was assayed using the B9 hybridoma cell line²⁶, and is expressed as a percentage of that observed in the presence of LPS alone. Results of two representative experiments are shown. Recombinant IL-13 protein was produced by secretion either in *S. cerevisiae* using a yeast expression plasmid containing the prepro sequence of pheromone- α (ref. 27), or in stably transfected Chinese hamster ovary cells. It was purified by ion-exchange chromatography on an S-fast flow column (Pharmacia) using fixation at pH5, and stepwise elution with increasing NaCl concentrations (with IL-13 eluting at 160 mM NaCl), and gel filtration on high-resolution Sephacryl-100 (Pharmacia). **b**, Inhibition of monokine mRNA accumulation in PBMC. Levels of IL-1 β , IL-6 and TNF- α mRNAs in adherent PBMC incubated for 4 h with 5 μ g ml⁻¹ LPS and 10 or 20 ng ml⁻¹ IL-13 are expressed as a percentage of the level obtained in the presence of LPS alone. Hybridization intensities on blots were analysed using the ImageQuant program on a Phosphorimager (Molecular Dynamics). Results are the means and standard deviations of 5 (TNF- α) or 6 (IL-1 β , IL-6) independent experiments. **c**, Modulation of IFN- γ synthesis in large granular lymphocytes (LGL). Interferon- γ synthesis in LGL isolated from peripheral blood leukocytes as described²⁸, and incubated in the presence of 25 ng ml⁻¹ IL-13, 10 ng ml⁻¹ IL-4, and/or 50 or 500 U ml⁻¹ of IL-2, is indicated in units per ml. Culture supernatants at 60–72 h were analysed for IFN- γ using a solid-phase ELISA assay (Genzyme). Results of two representative experiments are presented.

secreted by transfected COS or Chinese hamster ovary cells begins at a glycine residue at position 21; the non-glycosylated form has a molecular mass of 12,397 by electrospray mass spectrometry, close to the predicted M_r of 12,399, although it migrates as a 9K protein on SDS-PAGE (Fig. 1C).

The production by monocytes of inflammatory cytokines such as IL-1, IL-6 and tumour necrosis factor- α (TNF- α) is a crucial initiating event in a number of infectious and inflammatory pathologies. We have found that IL-13 strongly inhibits IL-6 secretion induced by bacterial lipopolysaccharide (LPS) in peripheral blood mononuclear cells (PBMC) (Fig. 3a). IL-13 would appear to be acting directly on monocytes because an inhibition of IL-6 mRNA accumulation is observed rapidly (within 4 hours) in cultures of PBMC enriched in monocytes by adherence to tissue culture dishes (Fig. 3b). A marked inhibition is also seen for other inflammatory monokine mRNAs (IL-1 β , TNF- α , IL-8, gro- β) in LPS-treated monocytes in the presence of IL-13 (Fig. 3b, and other data not shown). The action of IL-13 would thus seem to be a generalized block on inflammatory monokine synthesis, a property shared with the other Th2 lymphokines IL-4 and IL-10 (ref. 13). IL-13 and IL-4 show similar levels of inhibition of IL-6 synthesis (Fig. 3a). IL-13 also inhibits production of human immunodeficiency virus (HIV) by tissue-culture differentiated macrophages¹⁴, contrasting with the stimulatory effects of macrophage-activating cytokines such as IL-3 and GM-CSF on HIV production that have been reported in comparable conditions¹⁵.

The production of IFN- γ by large granular lymphocytes (LGL) may direct subsequent immune responses, leading to macrophage activation and to a 'Th1-type' cellular immune response¹⁶. The major cytokine influencing this production of IFN- γ is IL-2 (ref. 17). We have found that IL-13 has a small, direct effect on IFN- γ synthesis by LGL, and synergizes with both suboptimal and optimal doses of IL-2 (Fig. 3c). In this respect it resembles IL-12 (ref. 17), rather than IL-4, which strongly inhibits IFN- γ synthesis by these cells (Fig. 3c).

IL-13 also affects B lymphocytes, increasing their proliferation and the expression of the CD23 surface antigen (P. Carayon and T. Defrance, personal communication). IL-13 is thus a highly pleiotropic cytokine. In its anti-inflammatory effects on monocytes and its stimulation of the humoral response through B lymphocytes, IL-13 contributes to the 'Th2-type' response together with IL-4 and IL-10 (refs 18, 19). In, however, its effects on IFN- γ synthesis, it might be expected to promote a 'Th1-type' cellular immune response^{16,20}. A full understanding of the cytokine network in different pathological situations now needs to take into account the activities of IL-13.

The anti-inflammatory function of IL-13 may be crucial in clinical inflammation, for example in septic shock²¹ or rheumatoid arthritis²². Its activity on LGL may be clinically interesting in that, unlike IL-4, it does not decrease and can even increase the IL-2-induced lymphokine-activated killer activity of these cells (our unpublished results). As IL-13 also inhibits HIV replication *in vitro*¹⁴, and systemic immunity to parental tumour cells can be induced by IL-13-secreting tumour cells *in vivo* (D. Fradelizi, personal communication), IL-13 would appear to represent a potentially important new member of the therapeutic cytokine arsenal. □

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Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy

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CYSTIC FIBROSIS (CF) is a lethal inherited disorder affecting about 1 in 2,000 Caucasians. The major cause of morbidity is permanent lung damage resulting from ion transport abnormalities in airway epithelia that lead to mucus accumulation and bacterial colonization. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene¹ that encodes a cyclic-AMP-regulated chloride channel^{2,3}. Cyclic-AMP-regulated chloride conductances are altered in airway epithelia from CF patients⁴⁻⁶, suggesting that the functional expression of CFTR in the airways of CF patients may be a strategy for treatment. Transgenic mice⁷⁻⁹ with a disrupted *cftr* gene are appropriate for testing gene therapy protocols. Here we report the use of liposomes to deliver a CFTR expression plasmid to epithelia of the airway and to alveoli deep in the lung, leading to the correction of the ion conductance defects found in the trachea of transgenic (*cf/cf*) mice. These studies illustrate the feasibility of gene therapy for the pulmonary aspects of CF in humans.

Plasmid DNA complexed with cationic liposomes can be successfully delivered and expressed in airway epithelia of rodents^{10,11}. A suitable plasmid for expressing CFTR protein was constructed in the vector pREP8 (see legend to Fig. 1). In this plasmid, pREP8-CFTR, the human CFTR complementary DNA is under transcriptional control of the constitutive Rous sarcoma virus (RSV) 3' long terminal repeat (LTR) promoter, known to be active in nonproliferating airway epithelial cells¹⁰. To show that pREP8-CFTR expresses CFTR protein after

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transfection, plasmid DNA complexed with cationic liposomes was introduced into HeLa cells and CFTR protein detected by western blotting (Fig. 1a). To ascertain whether the expressed CFTR protein was functional, cAMP-stimulated iodide efflux was measured in transfected cells (Fig. 1b). In HeLa cells transfected with pREP8-CFTR, iodide efflux was stimulated by a cAMP-agonist cocktail. The cocktail did not stimulate efflux from cells transfected with the vector pREP8. The characteristics of this cAMP-stimulated anion efflux were similar to those reported previously for CFTR-expressing cells^{12,13}. Thus, cells transfected with pREP8-CFTR express functional CFTR protein.

RNA *in situ* hybridization was used to demonstrate that the CFTR expression plasmid can be delivered to airway epithelial cells by liposome-mediated transfection *in vivo*. Because CFTR messenger RNA is expressed at high levels in human and rodent intestinal crypts¹⁴⁻¹⁶, mouse intestinal sections were used as controls to demonstrate probe specificity. No hybridization to mouse intestine was detected with either of two human CFTR probes whereas, in consecutive sections, the mouse antisense *cftr* probe (but not the sense probe) detected abundant *cftr* mRNA in the crypts (data not shown). Additionally, neither the antisense nor the sense *hisD* vector control probes hybridized with mouse intestinal mRNA, as expected (data not shown). This demonstrates that the human CFTR and vector *hisD* probes do not cross-hybridize with mouse *cftr* mRNA.

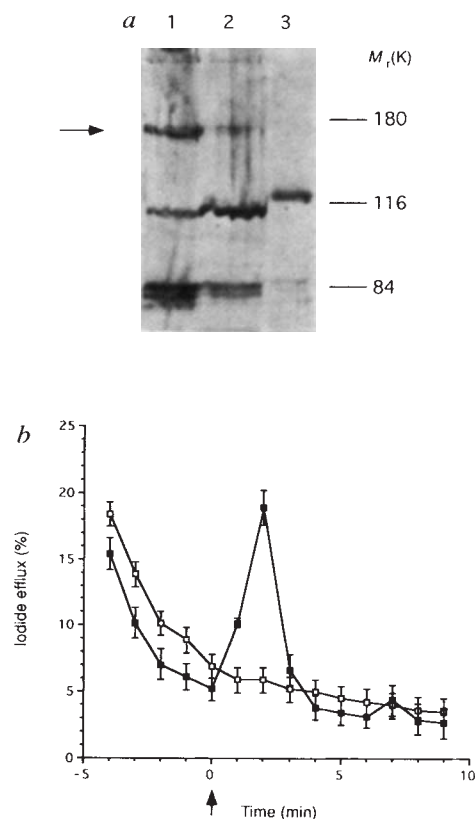
After transfection of pREP8-CFTR DNA into the airways of mice of 20-28 days old, sequences corresponding to human CFTR were detected by *in situ* hybridization (Fig. 2). Strong hybridization signals were observed in isolated groups of airway cells using both the human CFTR probe (Fig. 2a-c) and the

hisD vector-specific probe (d-f). In a series of consecutive sections the hybridization signals observed with the human CFTR and *hisD* probes colocalized to the same airways and airspaces (Fig. 2a-f). No hybridization was detected with the mouse *cftr* probe. This provides strong evidence that the hybridization signals obtained are highly specific and due to the transfected plasmid. Hybridization of these same probes to lung sections from untransfected animals served as a negative control against nonspecific hybridization; neither the human CFTR probe (g-i) nor the *hisD* probe (j-l) hybridized to any mRNAs in the lungs of untransfected mice. Hybridization signals were obtained with both the sense and antisense probes (Fig. 2a-f). Normally the antisense probe is used to detect mRNA whereas the sense probe serves as a negative control. Following transfection, however, both the sense and antisense probes would be expected to recognize vector DNA. The stronger signal observed with the antisense probe indicates transcription. This was seen for the *hisD* gene which is transcribed from a vector promoter. In most hybridizing cells, the signal obtained with the antisense human CFTR probe (Fig. 2b) was also greater than that obtained with the sense probe (c), implying that human CFTR mRNA is expressed following transfection.

The data in Fig. 3 show hybridization to sections through different regions of the lungs of a mouse which had been transfected with pREP8-CFTR. No expression of endogenous mouse *cftr* mRNA was detected in any region of the lung (data not shown), consistent with previous studies showing low-level *cftr* expression in rodent lung¹⁴, and with detailed studies of these transgenic animals (A.E.O.T. *et al.*, manuscript in preparation). This shows that the transfection protocol does not induce expression of endogenous mouse *cftr* mRNA. Human CFTR

FIG. 1 Expression of functional CFTR protein from plasmid pREP8-CFTR in HeLa cells. **a**, Western blot confirming expression of CFTR after transfection of HeLa cells using Lipofectin. Lanes 1, HT29 cells; 2, HeLa cells transfected with pREP8-CFTR; 3, HeLa cells transfected with the vector pREP8. The HT29 cells served as a positive control for CFTR expression and migration^{25,26}, indicated by the arrow. The ~115 and 85K bands are due to nonspecific cross reactions of the antibody²⁵. $M_r \times 1,000$ of markers is indicated. **b**, Time course of iodide efflux from HeLa cells. Cells were transfected with plasmid pREP8-CFTR (■) or the vector pREP8 (□). The arrow indicates the point at which a cAMP-agonist cocktail was added. The data are displayed as the mean of three individual experiments ($\pm$ s.e.m.), expressed as a percentage of the total efflux.

METHODS. Human CFTR cDNA encoding the entire CFTR coding sequence¹ (nucleotides 133-4,620) was inserted into the plasmid pREP8 (Invitrogen), under transcriptional control of the RSV 3' LTR promoter, to create plasmid pREP8-CFTR. The cDNA incorporated three minor changes from the published sequence (C to G at nucleotide 136¹³; T to C at nucleotide 936²⁷; A to C at nucleotide 1990²⁸), and included a Kozak translation initiation sequence²⁹ (CCACCATG) immediately 5' to the translation initiation codon. For plasmid transfection, 1×10^6 HeLa cells were seeded into each well of 35-mm, 6-well tissue culture dishes in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, and incubated at 37 °C. After 24 h growth, cells in each well were transfected with 8 μ g plasmid DNA mixed with 13 μ g Lipofectin (Gibco BRL) and diluted to 3 ml in Optimem 1 (Gibco BRL). After a further 24-48-h incubation at 37 °C, cells were either collected for protein extraction or used for anion efflux measurements. For protein extraction, cells were washed five times with ice-cold PBS and collected into a buffer containing 10 mM Tris-Cl pH 8.0, 10 mM KCl, 1.5 mM MgCl₂, and the protease inhibitors antipain (50 μ g ml⁻¹), aprotinin (10 μ g ml⁻¹), benzamide (310 μ g ml⁻¹), leupeptin (5 μ g ml⁻¹), pepstatin A (5 μ g ml⁻¹) and phenylmethylsulphonyl fluoride (175 μ g ml⁻¹). Cells were lysed by repeated passage through a 19-gauge needle. Cellular and nuclear debris were removed from the lysate by a 5-min centrifugation at 300g and membranes pelleted by a 30-min centrifugation at 100,000g. The membrane pellet was dissolved in 2.5% Triton X-100 and separated by electrophoresis on a 6% SDS-polyacrylamide gel. CFTR was detected by western blotting after transfer to a Hybond C-super membrane (Amersham) using the well characterized anti-CFTR antisera 181²⁵. Immunodetection was by enhanced chemiluminescence (ECL; Amersham). To measure cAMP-stimulated efflux, the transfected cells were preloaded with iodide by incubation for 40 min at room temperature in 3 ml loading buffer (136 mM NaI, 3 mM KNO₃, 2 mM Ca(NO₃)₂, 11 mM glucose, 20 mM HEPES, pH 7.4). Extracellular NaI was removed by



6 $\times$ 1 ml rinses in efflux buffer (loading buffer with 136 mM NaNO₃ replacing the NaI). Cells were then washed with 1 ml efflux buffer for 1 min using a sample-replace procedure. After the fifth 1-min sample (designated time 0), cAMP-agonists (1 mM 3-isobutyl-1-methylxanthine (IBMX), 200 μ M dibutyryl-cAMP, 10 μ M forskolin, dissolved in DMSO) were included in the efflux buffer. The concentration of iodide in each 1-ml aliquot was determined using an iodide-specific electrode (HNU systems).

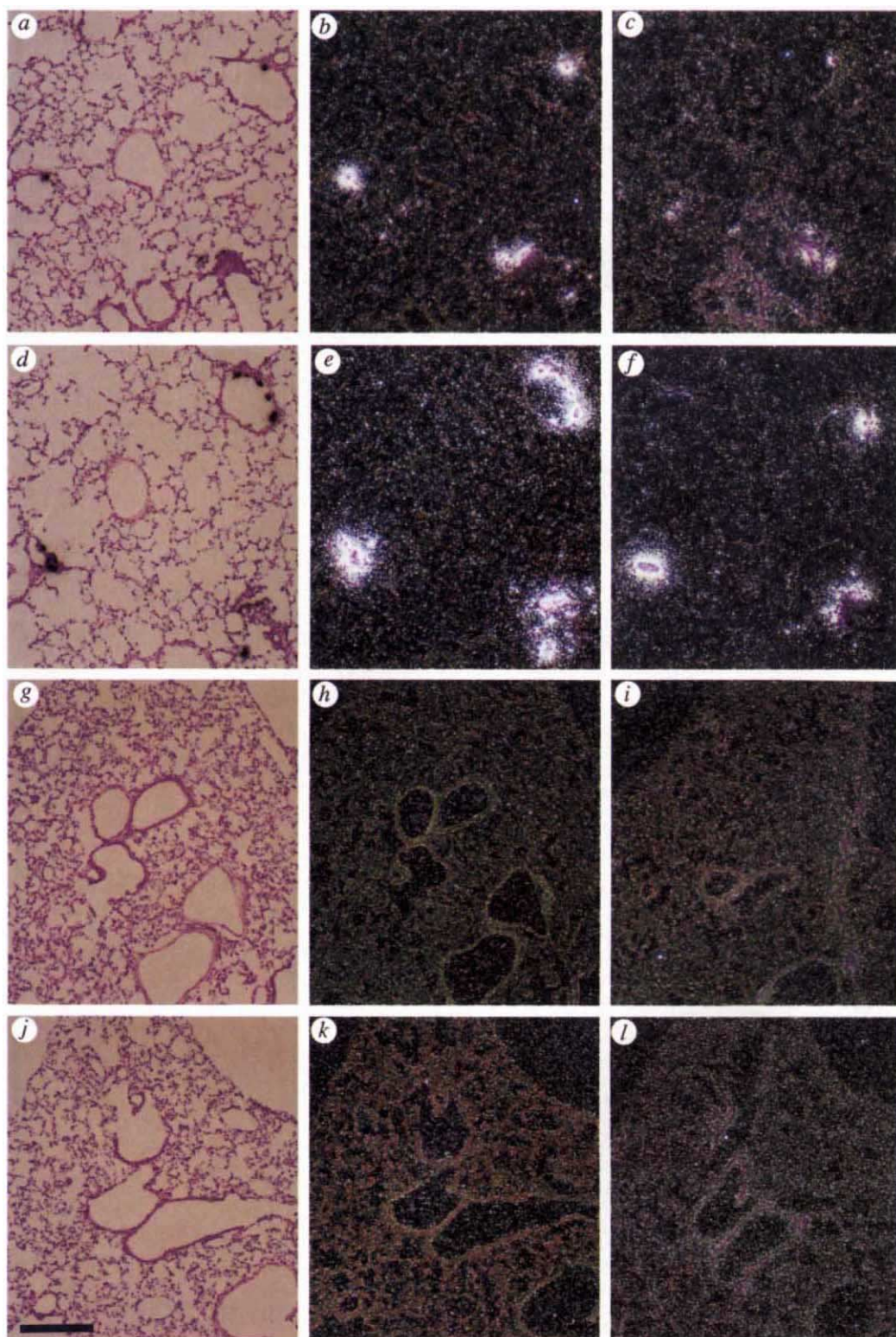
expression was seen in the airways of three out of four animals transfected with pREP8-CFTR and, in at least one transfected animal, human *CFTR* sequences were detected in all five lobes of the lung. Positive cells were detected in large and small airways (Fig. 2*a-d*), and in cells lining the air spaces of the more distal regions of the lung (*e-j*). It appeared to be the surface epithelial cells of the airways that had been transfected. Colocalization of the *CFTR* signal with the *hisD* probe (Fig. 2), confirmed that the signal was a consequence of transfection.

FIG. 2 Detection of human *CFTR* by *in situ* hybridization in mouse airways following *in vivo* transfection. *a-f*, Data obtained for a mouse transfected with pREP8-CFTR; *g-l*, controls for an untransfected mouse. The probes used were against human *CFTR* exons 1-6 (*a-c* and *g-i*) or the *hisD* vector sequences (*d-f* and *j-l*). For each example, three panels are shown: (1) a brightfield view of a section hybridized with the antisense probe, to illustrate tissue morphology (*a, d, g, j*); (2) a darkfield view of the same section (*b, e, h, k*); (3) a darkfield view of an adjacent section probed with the control sense probe (*c, f, i, l*). Scale bar, 200 μ m. Similar results were obtained with several animals.

METHODS. Mice were given enough avertin by intraperitoneal injection to induce very light anaesthesia. For transfection, ~100 μ g plasmid DNA was mixed with 25 μ g Lipofectin in a total volume of 50 μ l and administered to mice by tracheal instillation in two loads by insertion of a metal applicator, adapted from a 25-gauge blunted syringe needle, through the mouth and into the trachea to the point where the main bronchi branch off. The animals used weighed between 5 g and 12 g. Four days after transfection, *in situ* hybridization was performed on perfusion-fixed tissue by a modification of the method described by Simmons *et al.*³⁰, as described previously¹⁵. ³⁵S-labelled RNA probes were synthesized *in vitro* by run-off transcription from plasmid DNA, incorporating [³⁵S]UTP. The antisense and sense (control) probes were derived from opposite strands of the same plasmid. The plasmids used for probe generation were as follows. The two human *CFTR* probes, corresponding to nucleotides 62-645 (exons 1-6) and nucleotides 1,977-2,461 (exon 13) (numbering according to ref. 1), have been described previously¹⁶. The mouse *cfr* probes were derived by reverse transcriptase PCR from mouse testis mRNA and corresponded to nucleotides 305-691 of exons 3-5. The *hisD* vector probe was subcloned from pREP8 and corresponded to nucleotides 3,167-3,851. All probes were cloned into Bluescript vectors (Stratagene). After developing,

Thus, transfection is effective and expression of human *CFTR* throughout the airway was achieved.

To determine whether delivery of *CFTR* cDNA to the airways could correct the ion transport defects apparent in CF, we used a recently developed mouse model^{9,17}. These transgenic (*cf/cf*) mice are homozygous for a null mutation in *cfr* and express little or no detectable endogenous *cfr* mRNA (A.E.O.T. *et al.*, manuscript in preparation). *CFTR*-dependent, cAMP-stimulated chloride conductances are greatly reduced in the airways



sections were counterstained with haematoxylin and eosin and photographed using a Nikon Microphot FX microscope.

and caeca of these mice, compared with normal (+/+) animals, mimicking features of the human disorder¹⁷. The mice frequently die shortly after birth as a consequence of intestinal blockages⁹. Ion transport in the trachea was measured by voltage clamping at zero potential, using pharmacological agents to eliminate or stimulate various processes (Fig. 4). Measurements were also made with the caecum of the same animal as an internal control. Figure 4A shows a set of typical results, and Fig. 4B a compilation of the data. For each tracheal preparation, three measurements were made: amiloride-sensitive sodium absorption (labelled Na^+), cAMP-stimulated chloride secretion (labelled Cl^- cAMP), and Ca^{2+} -stimulated chloride secretion (labelled Cl^- Ca^{2+}). As expected, CFTR-dependent, cAMP-stimulated

chloride secretion was significantly reduced ($P < 0.01$) in both the tracheas and caeca of the *cf/cf* mice compared with the normal (+/+) mice. There was no significant difference in the cAMP-stimulated chloride secretion between untreated and pREP8-transfected normal mice, indicating that transfection itself has no effect on ion transport. Most importantly, transfection of *cf/cf* mice with pREP8-CFTR restored the cAMP-stimulated chloride secretion in the trachea to a level comparable with that of normal (+/+) animals. In sharp contrast, transfection of the *cf/cf* mice with the vector pREP8 had no significant effect on the cAMP-stimulated chloride secretion in the trachea. The caecum of *cf/cf* mice transfected with pREP8-CFTR showed no appreciable cAMP-stimulated chloride secretion

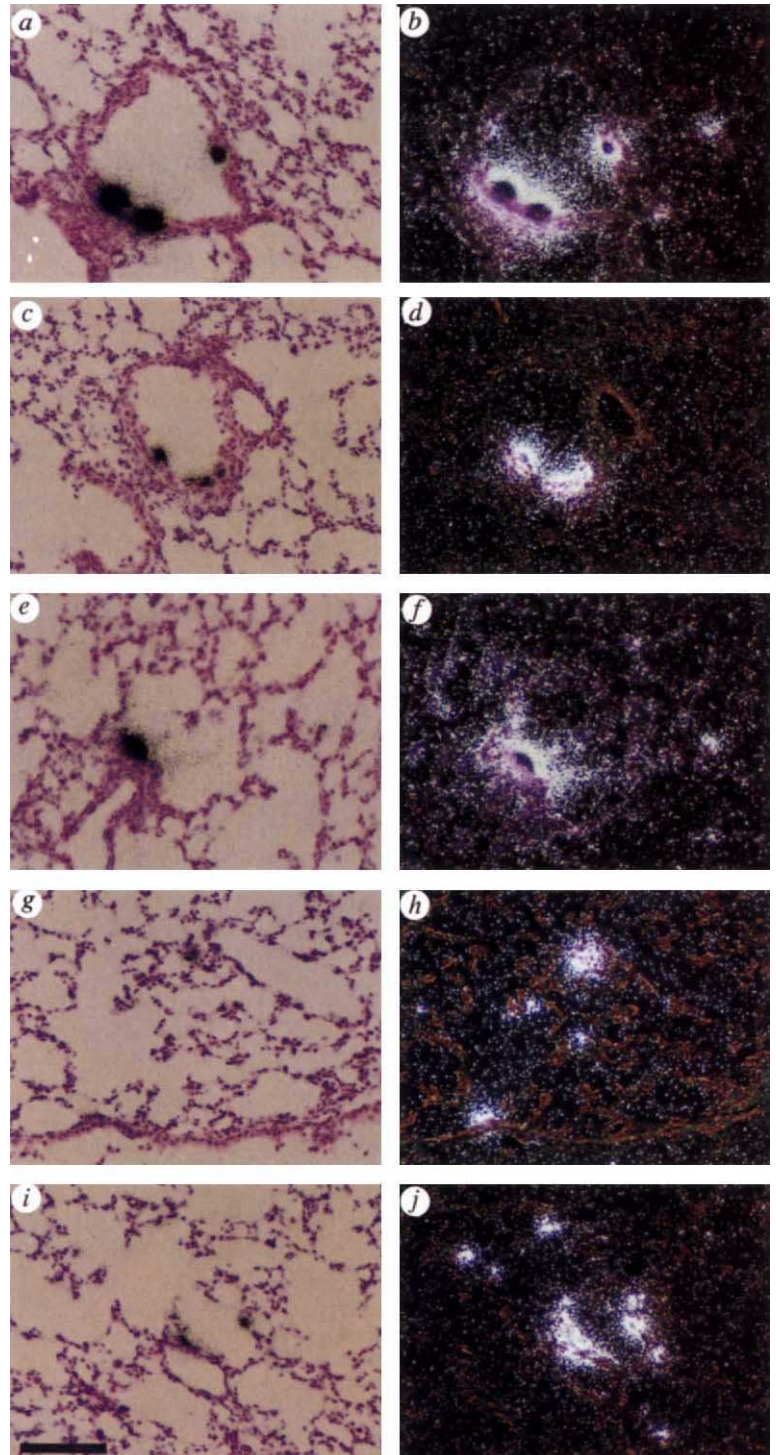
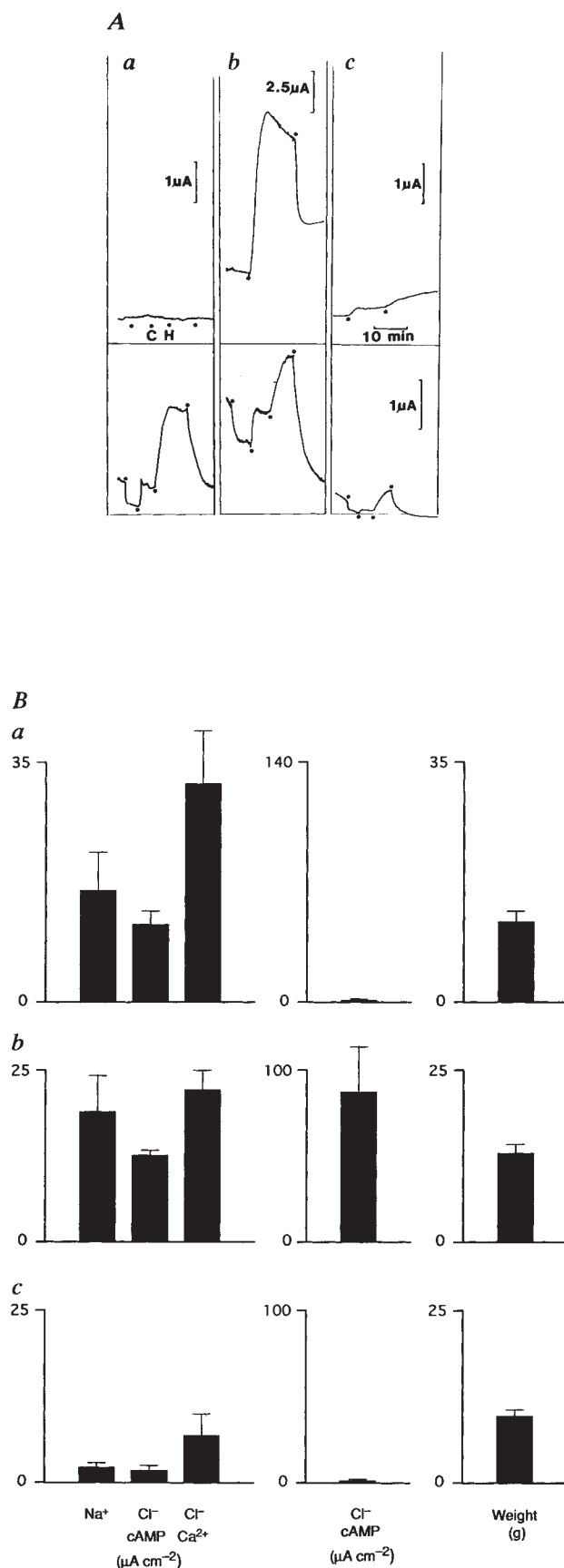


FIG. 3 Detection of human CFTR in different regions of the mouse airway following transfection. Sections from different regions of the airway of a mouse transfected with pREP8-CFTR were hybridized with human antisense *CFTR* probes corresponding to either exons 1–6 (*a–f, i, j*) or exon 13 (*g* and *h*). *a–d*, Expression of human *CFTR* in small and large airways; *e–j*, expression in airspaces in the more distal regions of the lung. Some variation was found in the proportion of cells transfected in different animals which probably reflects differences in the amounts of DNA delivered. Scale bar, 100 μm . See legend to Fig. 2 for details of methods and hybridization probes.

FIG. 4 Correction of the ion channel defects in the trachea of transgenic (*cf/cf*) mice. **A**, Sample traces showing examples of the data from which panel **B** was compiled. Three paired tracheal/caecal preparations are shown. Upper records show measurements for the caecum and lower records for the trachea of the same animal. *a*, *cf/cf* mouse transfected with pREP8-CFTR; *b*, *+/+* mouse transfected with pREP8; *c*, *cf/cf* mouse transfected with the vector pREP8. Four additions were made to each of the tracheal preparations at the time points indicated by dots. First, amiloride (100 μ M) was added (apically) to block electrogenic sodium absorption and to ensure subsequent current increases were not due to this activity. EC_{50} for amiloride is ~ 1 μ M and 100 μ M will give essentially 100% inhibition³¹. Second, forskolin (10 μ M) was added to both sides of the membrane to stimulate adenylate cyclase and activate cAMP-sensitive chloride channels, increasing chloride secretion. Third, the Ca^{2+} ionophore A23187 (1 μ M) was added to both sides of the membrane to activate Ca^{2+} -dependent chloride secretion. The ionophore-induced responses were much slower than those induced by forskolin. Finally, frusemide (1 mM) was added basolaterally to block chloride secretion, confirming the nature of the electrogenic transporting activity. Frusemide (1 mM) inhibits over 90% of Cl^{-} secretion¹⁷. Calibrations for the trachea are the same in each panel. Caecal preparations (upper records) received two additions, forskolin (10 μ M, added to both sides of the membrane) and frusemide (1 mM, added basolaterally). Other additions are specifically labelled: C, carbachol (10 μ M); H, histamine (10 μ M). In both the caecum and the trachea the chloride secretory responses were inhibited by frusemide, indicating that they are due to electrogenic chloride secretion from the basolateral to the luminal side of the epithelium. In 8 out of 10 *cf/cf* caeca, frusemide led to a slight increase in short-circuit current (SSC) (*c*, upper record); this is probably due to a blockage of K^{+} secretion and is typical of the caecum of *cf/cf* mice¹⁷. **B**, Compilation of the data. Mice were subjected to three different treatment protocols: *a*, *cf/cf* mice transfected with pREP8-CFTR; *b*, *+/+* mice transfected with the vector pREP8; *c*, *cf/cf* mice transfected with the vector pREP8. Four animals in each group were matched based on a compromise between weight and age. Data were only included when paired airway and caecal measurements could be made for the same animal. The genotypes of the transfected mice, and of the plasmid DNA with which they were transfected, was unknown at the time the measurements were made. For each treatment regime, three sets of data are shown. (1) The left hand columns show SCC measurements for the trachea. Three measurements of SCC changes are presented: Na^{+} , amiloride-sensitive sodium absorption³¹; Cl^{-} cAMP, SCC change induced by forskolin, presumed to reflect CFTR function^{17,32}; Cl^{-} Ca^{2+} , SCC change induced by the addition of the calcium ionophore A23187. As about 50% of the basal current in the airways was due to sodium absorption, chloride secretion was measured after the addition of amiloride (100 μ M) which abolished electrogenic sodium absorption. (2) The central columns show SCC measurements for the caecum. Only cAMP-sensitive chloride secretion (Cl^{-} cAMP), induced by the addition of forskolin (10 μ M), was measured. Amiloride was not added because the caecum shows no sodium absorptive current^{17,33}. (3) The right-hand columns show the weights of the animals used (mean $\pm$ s.e.m.). Note: the ion transport characteristics of 4/6 pREP8-CFTR transfected *cf/cf* mice were altered by transfection; the reason for the failure of the other two mice is almost certainly failure in delivery. Nevertheless, the forskolin-sensitive SCC (Cl^{-} cAMP) in the whole group including the two failures (9.2 ± 2.6 $\mu A cm^{-2}$, $n=6$) was significantly greater ($P < 0.05$, Mann and Whitney test) than the value for *cf/cf* mice (1.9 ± 0.5 $\mu A cm^{-2}$, $n=4$). Finally, data for two other groups of animals were obtained although these are not illustrated in the figure. Untreated, wild-type (*+/+*) mice ($n=5$; weight $\pm$ s.e.m. = 32.2 ± 2.9 g) had transport parameters as follows (mean $\pm$ s.e.m.): for the trachea: Na^{+} = 10.7 ± 4.8 $\mu A cm^{-2}$, Cl^{-} cAMP = 11.4 ± 4.3 $\mu A cm^{-2}$, Cl^{-} Ca^{2+} = 12.1 ± 4.7 $\mu A cm^{-2}$; for the caecum Cl^{-} cAMP = 35.6 ± 7.0 $\mu A cm^{-2}$. Heterozygous (*cf/+*) mice transfected with pREP8-CFTR ($n=2$; mean weight, 7.0 g) had the following transport parameters (mean $\pm$ s.e.m.): for the trachea: Na^{+} = 4.5 $\mu A cm^{-2}$, Cl^{-} cAMP = 6.6 $\mu A cm^{-2}$, Cl^{-} Ca^{2+} = 8.2 $\mu A cm^{-2}$; for the caecum Cl^{-} cAMP = 104.6 $\mu A cm^{-2}$. Note that the forskolin-sensitive currents (Cl^{-} cAMP) in the trachea were smaller than those reported previously for wild-type mice¹⁷. This is undoubtedly a consequence of edge damage caused by using only 2.27 mm² areas of trachea in the present study, necessitated by the small size of the *cf/cf* mice, compared with 4 mm² areas of trachea in previous studies.

METHODS. Transgenic mice were genotyped by PCR and/or Southern blot analysis as described¹⁷. Introduction of plasmid DNA into the mouse airways was as described in the legend to Fig. 2. Trachea and caeca were removed from the transfected animals killed by exposure to 100% CO_2 . A single tracheal preparation (2.27 mm²) and a single caecal preparation (20 mm²) was prepared from each animal. The reduction in tracheal area, compared with a previous report¹⁷ was due to the necessity of using animals as small as 5 g. The trachea were cleaned and cut longitudinally along the dorsal



surface and a piece placed under microscopic control in a specially constructed Ussing chamber designed to preserve the curvature of the tissue. Electrogenic ion transport was measured directly as SCC recorded by voltage clamping the tissue at zero potential, as described previously¹⁷.

compared with the control (+/+) mice, confirming the genotypes of the mice and that the transfection procedure did not affect the gut. Thus, the transfection procedure used can restore CFTR-dependent, cAMP-stimulated chloride secretion by airway epithelia to normal levels.

In the airways of human CF patients there is an increase in amiloride-sensitive sodium absorption, as well as a decrease in chloride secretion, compared with controls¹⁸⁻²⁰. It has been suggested that this is crucial to the development of the disease state, as application of amiloride by aerosol alleviates the decline in lung function in CF^{21,22}. It is not yet clear how a loss of CFTR function leads to this increase in sodium absorption. In contrast to the human, sodium absorption was reduced in the airways of *cf/cf* mice (Fig. 4B). Transfection of the *cf/cf* mice with pREP8-CFTR, but not with the vector pREP8, significantly increased sodium absorption (seven- to eightfold; $P < 0.05$), to essentially wild-type (+/+) levels (Fig. 4B). Thus, secondary alterations in sodium transport were also corrected to wild-type levels by the transfection protocol used. Finally, Ca^{2+} -induced chloride secretion reflects an alternative pathway for chloride secretion in the airways distinct from the CFTR pathway^{4,23}. Ca^{2+} -stimulated chloride secretory currents were not defective in *cf/cf* trachea, compared with trachea of normal (+/+) mice, but were significantly increased following transfection with CFTR ($P < 0.05$; Fig. 4B). This latter increase is probably a consequence of hyperpolarization through Ca^{2+} -sensitive K^{+} channels, which increases the electrochemical gradient for Cl^{-} exit through the introduced CFTR channels and the pre-existing second pathway²⁴.

These data show that the ion transport defects in CF can be corrected *in vivo*. Liposomes, which in clinical trials have been shown to be non-toxic and non-immunogenic, may be safer than viral vectors which have the inherent risks of immunogenicity, replication and transmission. Our results illustrate the invaluable role of transgenic null *cf/cf* mice in assessing the efficiency of various gene therapy approaches. We have shown that functional expression of CFTR not only corrects the primary ion transport defect of the trachea (that is, the cAMP-stimulated chloride secretion), but also corrects secondary alterations in sodium absorption which are a consequence of loss of CFTR function. There seems to be no reason why this approach should not be transferable to humans for the treatment of the pulmonary features of CF. □

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Germ-line transmission and expression of a human-derived yeast artificial chromosome

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INTRODUCTION of DNA fragments, hundreds of kilobases in size, into mouse embryonic stem (ES) cells would greatly advance the ability to manipulate the mouse genome. Mice generated from such modified cells would permit investigation of the function and expression of very large or crudely mapped genes. Large DNA molecules cloned into yeast artificial chromosomes (YACs) are stable and genetically manipulable within yeast¹, suggesting yeast-cell fusion as an ideal method for transferring large DNA segments into mammalian cells. Introduction of YACs into different cell types by this technique has been reported²⁻⁸; however, the incorporation of yeast DNA along with the YAC has raised doubts as to whether ES cells, modified in this way, would be able to recolonize the mouse germ line⁵. Here we provide, to our knowledge, the first demonstration of germ-line transmission and expression of a large human DNA fragment, introduced into ES cells by fusion with yeast spheroplasts. Proper development was not impaired by the cointegration of a large portion of the yeast genome with the YAC.

Yeast spheroplasts, carrying yHPRT, a 670 kilobase (kb) YAC containing the human hypoxanthine phosphoribosyltransferase (HPRT) gene⁴, were fused with the HPRT-deficient ES cell line E14TG2a (ref. 9). Clones expressing the HPRT locus were selected in hypoxanthine/aminopterin/thymidine (HAT) medium (Fig. 1 legend) and expanded. The human HPRT gene was detected by hybridization in all ES cell clones analysed (not shown). The integration of additional human sequences was examined by comparing the *Alu* profile of 37 HAT-resistant (ESY) clones to that of yHPRT in yeast. Most, if not all, of the 30 *Alu* fragments characteristic of yHPRT were present and of similar relative intensity in over 90% of the ESY clones (Figs 1a, 3b). In clones with an incomplete *Alu* profile (such as ESY 8-5, Fig. 1a) only a few fragments were missing or altered in size. In most ESY clones, the *Alu* pattern appeared to be intact and without significant deletion, rearrangement or segmental amplification.

Integration of YAC vector sequences was investigated with vector arm-specific probes. A 4.5 kb *Hind*III fragment, detected by the right arm probe in yHPRT, was observed in 10 of 20 ESY clones (Fig. 1b). This vector arm was lost in eight ESY clones (for example ESY 3-1, 3-6, Fig. 1b) and rearranged in two (for example ESY 8-6, Fig. 1b). The left arm probe detected the 3 kb and 4.1 kb *Hind*III yHPRT fragments in 18 of 20 clones (Fig. 1c). In total, 8 of the 20 clones (such as ESY 5-2, 8-7, 7-3, Fig. 1a-c) contained complete *Alu* profiles and both intact YAC vector arms.

The structural integrity of yHPRT in ESY clones 5-2 and 8-7 was further evaluated by pulsed-field gel electrophoresis. In yeast carrying yHPRT, five *Sfi*I fragments of the following rough sizes were defined by different probes: 315 kb (*Alu*, left arm),

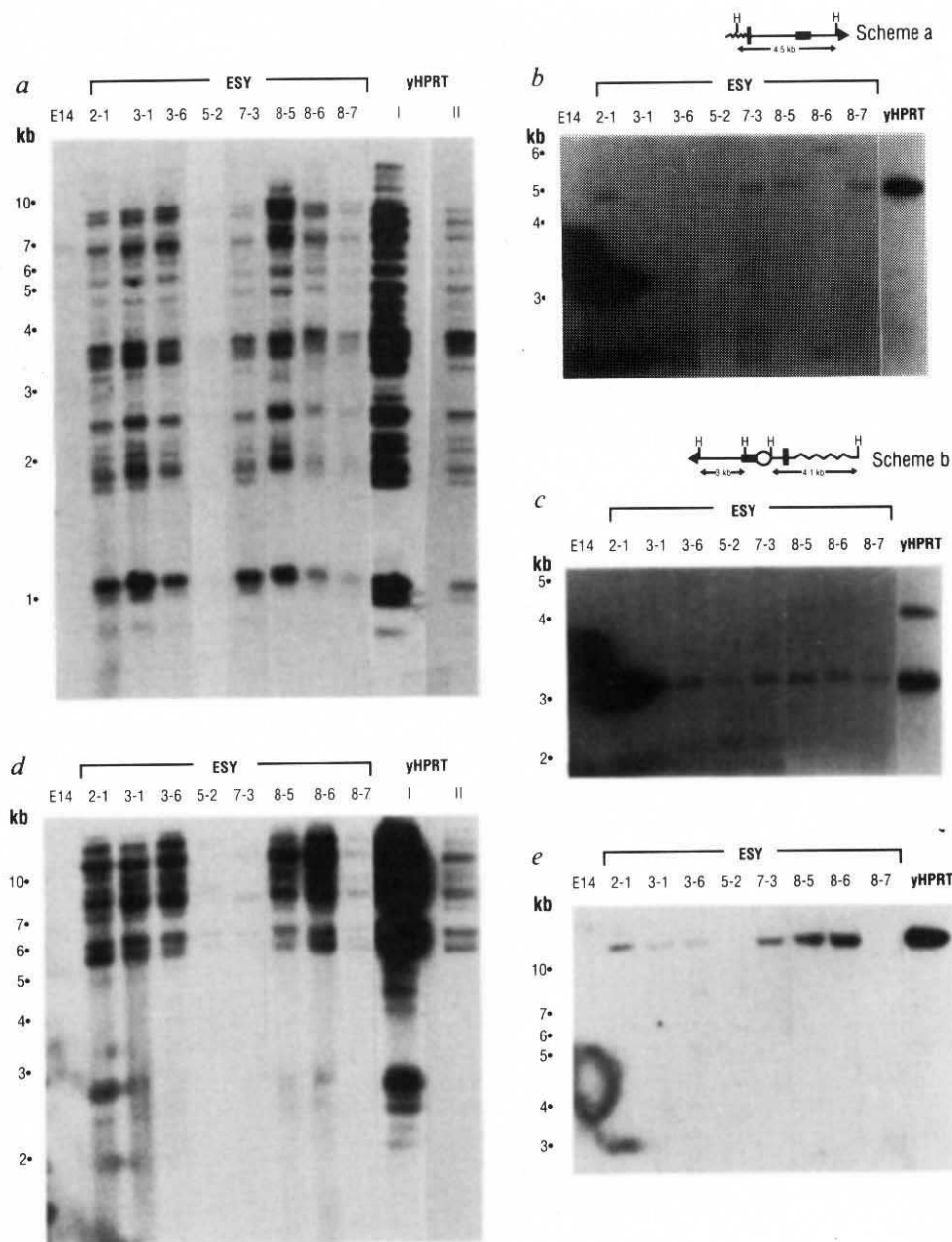
145 kb (*Alu*, HPRT), 95 kb (*Alu*, right arm), 70 and 50 kb (*Alu* only) (data not shown). In both ESY clones, the HPRT and *Alu*-specific fragments were similar in size to those from yHPRT. The end fragments detected for ESY 5-2 and 8-7 were larger than those from yHPRT, as expected for YACs integrated within a mouse chromosome (data not shown). These data, together with the *Alu* profiles, substantiate the structural integrity of the YAC in these clones. These studies were complemented by fluorescence *in situ* hybridization to ESY 8-7 (Fig. 2a, b) and ESY 8-6 (data not shown) metaphase chromosome spreads, in which a single integration site was detected for the human sequences.

The presence of yeast genomic DNA in the ESY clones was determined by hybridization with the repetitive element *Ty*, present in about 25 copies in this strain (Fig. 1d). The amount of integrated yeast DNA varied among the different clones and was not correlated with the portion of YAC DNA retained.

Some ESY clones (such as 3-1 and 8-6) appeared to contain a full *Ty* profile, whereas others (such as 5-2 and 8-7) contained only a fraction (20% or less) of these fragments. The yeast chromosomal *lys2* gene was present in all cell lines with a complete *Ty* profile (Fig. 1d) and absent in six of seven clones with an incomplete pattern. To determine if the yeast chromosomal DNA had integrated at single or multiple sites within the ES cell genome, fluorescent *in situ* hybridization was done on ESY clone 8-6 which contains a complete *Ty* profile (Fig. 1d). A single integration site was detected using a combined yeast repetitive probe (Fig. 2c, d), indicating that within the limits of resolution, all yeast DNA fragments are integrated in one block.

Using the ability of ES cells to undergo orderly *in vitro* differentiation, we investigated YAC stability and the effect of the integrated DNA on ESY cell pluripotency. Four clones, containing different amounts of yeast DNA (ESY 5-2, 3-6, 8-6 and 8-7), exhibited a differentiation pattern indistinguishable

FIG. 1 Characterization of yHPRT and yeast genomic DNA integrated in ESY clones. Southern blot analysis of *Hind*III-digested DNA from E14TG2a (20 µg), ESY clones: 2-1, 3-1, 3-6, 8-5, 8-6 (20 µg), 5-2, 8-7 (5 and 10 µg, respectively) and yHPRT-containing haploid yeast strain AB1380 (80 ng; owing to difference in genome size, an equivalent hybridization signal should be obtained from a single-copy sequence in 40 ng of haploid yeast DNA and in 20 µg of diploid mammalian DNA). Probes: a human *Alu* sequence (a); specific pBR322-derived right (b) and left (c) YAC vector arm probes; a yeast *Ty* repetitive sequence (d); the yeast single copy gene *lys2* (e). Two exposures (I, 48 h, II, 12 h) of yHPRT probed with *Alu* and *Ty* sequences are shown. Schemes of right (a) and left (b) vector arms and pBR322-derived YAC vector fragments are shown (◀, telomere; ■, yeast-derived sequences; ○, yeast centromere; —, pBR322-derived sequences; ~, human insert; ■, *Eco*RI cloning site; H, *Hind*III sites). METHODS. yHPRT-containing yeast cells¹⁰ (provided by C. Huxley) were spheroplasted using Zymolyase 20T (1.5 mg ml⁻¹) and fused with E14TG2a cells (cultured as in ref. 11) as described⁴. HAT selection was imposed 48 h postfusion. Thirty-seven HAT-resistant colonies (frequency of 0.5×10^{-6} per fused ES cell) were expanded for analysis. No colonies were detected in control ES cell/yeast spheroplasts fusion experiments. Four ESY clones analysed had normal karyotypes of 40 chromosomes (H. Tsuda, unpublished results). The human HPRT gene in ESY clones was detected with the human HPRT cDNA probe¹² (not shown). The number of integrated YACs per ESY cell generally was estimated to be one by relative hybridization intensity of the human HPRT locus in yeast and in the ESY clones (not shown). The *Alu* probe was a 300 bp *Bam*HI fragment from pBP63A (ref. 13). The right and left vector arm probes were pBR322-derived *Bam*HI-*Pvu*II 1.7 and 2.7 kb fragments, respectively (ref. 1, schemes a and b). The difference in the hybridization intensity of the 3- and 4.1-kb left arm bands correlates with the difference in the length of homology between these fragments and the probe. The yeast *Ty* repetitive probe was a 5.6 kb *Xho*I fragment¹⁴ from pJEF742 (provided by J. Boeke).



The *LYS2* probe was a 1.7 kb *Bam*HI fragment from pLUS (ref. 15). Using the relative hybridization intensities of the *lys2* gene in yeast and in ESY clones, we estimated its copy number per ESY cell generally to be one.

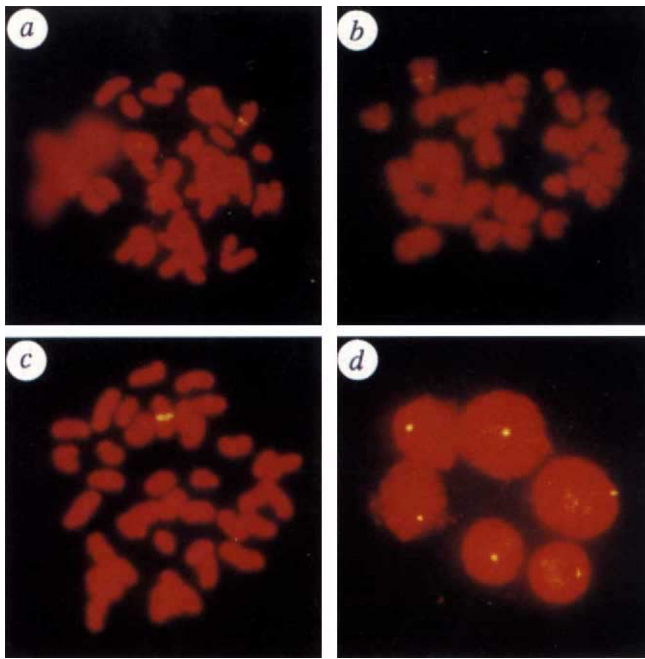


FIG. 2 Detection of a single integration site for yHPRT and for yeast genomic sequences in ESY cell chromosomes by *in situ* hybridization. Photomicrographs of representative metaphase spreads (*a, b, c*) or interphase nuclei (*d*) from ESY 8-7 cells (*a, b*) hybridized with biotinylated human genomic sequences, and ESY 8-6 cells (*c, d*) hybridized with biotinylated yeast repetitive DNA sequences.

METHODS. Biotinylated probes were prepared by nick translation in the presence of biotin-11-dCTP (Enzo Diagnostics) and biotin-14-dATP (Gibco-BRL). The human probe was generated from human genomic placental DNA (Clontech). The yeast probe consisted of a mixture of DNA fragments encoding the yeast repeated elements: *delta* (a 1.08 kb *Sau*3A fragment of p ϕ 6 (ref. 16)) and *Ty* (a 1.35 kb *Eco*RI-*Sal*I fragment of p29 (ref. 14)), both provided by P. Philippsen, the ribosomal DNAs (a 4.6 kb *Bgl*II L90 and a 4.4 kb *Bgl*II L92 fragment¹⁷) provided by S. Roeder, and the *Y'* elements (2.0 and 1.5 kb *Bgl*II-*Hind*III fragments of p198 (ref. 18)) provided by P. Hieter. Hybridization of sequences on chromosome spreads with biotinylated probes and detection by Avidin-FITC followed by biotin-anti-Avidin and Avidin-FITC amplification was done as described¹⁹, using a Zeiss Axiophot microscope. Chromosomes were counterstained with propidium iodide. The photomicrographs shown are representative of 95% of the metaphase spreads or interphase nuclei scanned in three independent experiments, done with the human or the yeast probes.

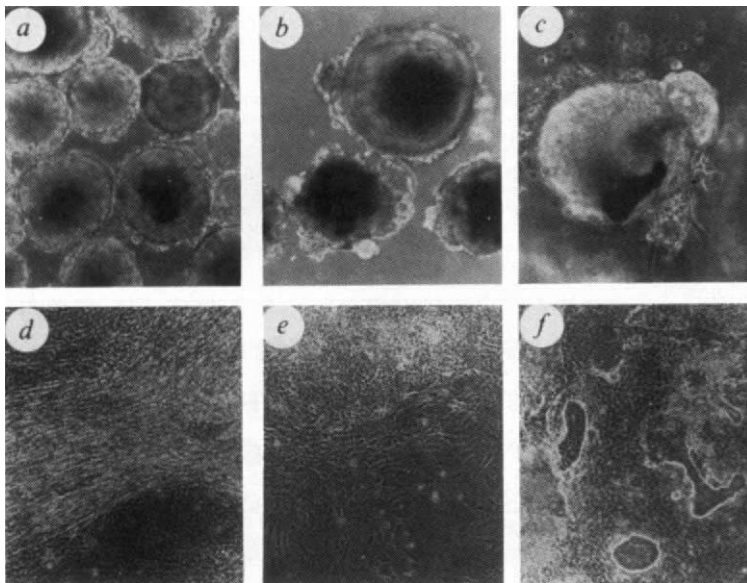
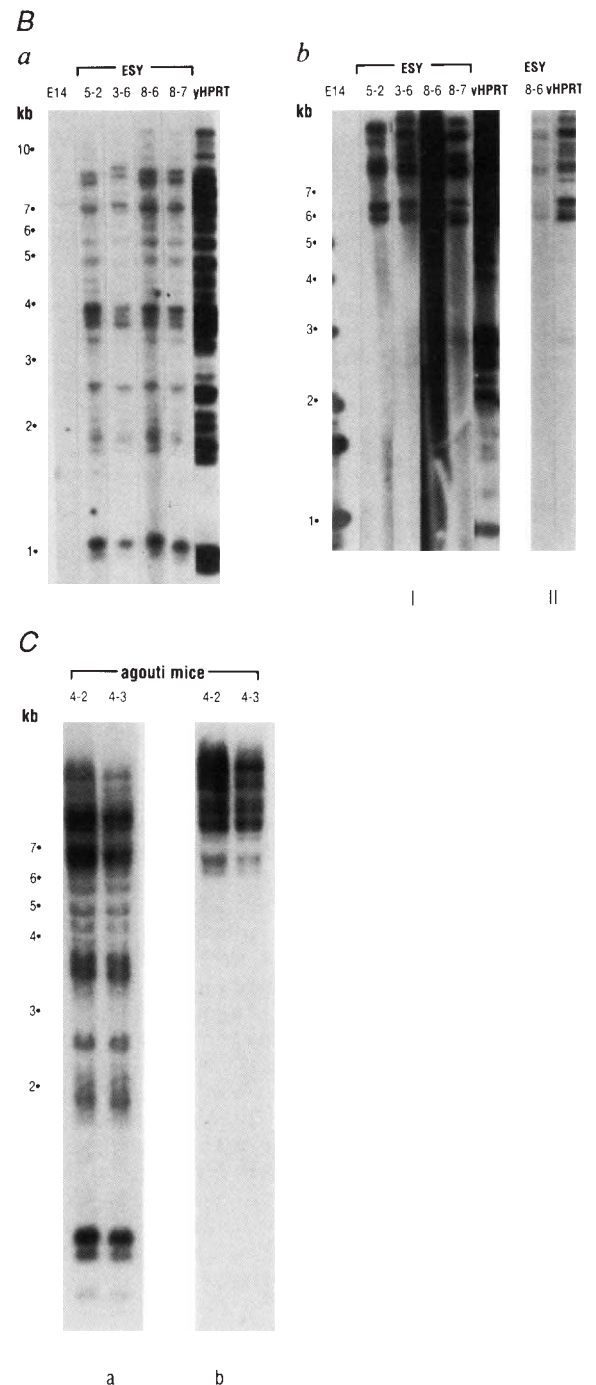


FIG. 3 yHPRT is stably retained during *in vitro* ES cell differentiation and is transmitted through the mouse germ line. *A*, Embryoid bodies (*a, b*) and differentiated cell types: blood islands (*c*), contracting muscle (*d*), neuronal cells (*e*) and neural tubules (*f*) formed by ESY clones. *B*, Southern blot analysis of DNA extracted from differentiated ESY 5-2, 3-6, 8-6, 8-7 (20 μ g) and yHPRT in AB1380 (40 ng) using human *Alu* probe (*a*) and yeast *Ty* sequences (*b*). Two exposures (I, 48 h, II, 12 h) of ESY 8-6 and yHPRT probed with *Ty* are shown. *C*, Southern blot analysis of tail DNA (20 μ g) from two agouti offspring (4-2 and 4-3) derived from ESY 3-1 chimaeric male 394/95-2 using human *Alu* (*a*) and yeast *Ty* (*b*) sequences.

METHODS. ESY clones were induced to form embryoid bodies and to give rise to differentiated cell types by culturing them as described²⁰. No difference in growth rate was observed when differentiated cultures, grown in the absence of selection for over 30 days, were transferred to HAT-selective medium. ESY clones were microinjected into C57BL/6J mouse blastocysts and chimaeric mice were generated as described²¹. Chimaeric males were mated with C57BL/6J females and germ-line transmission was determined by the presence of agouti offspring. Three of the ESY3-1-derived males, 394/95-1, 394/95-2 and 411-1, transmitted the ES cell genome to their offspring.



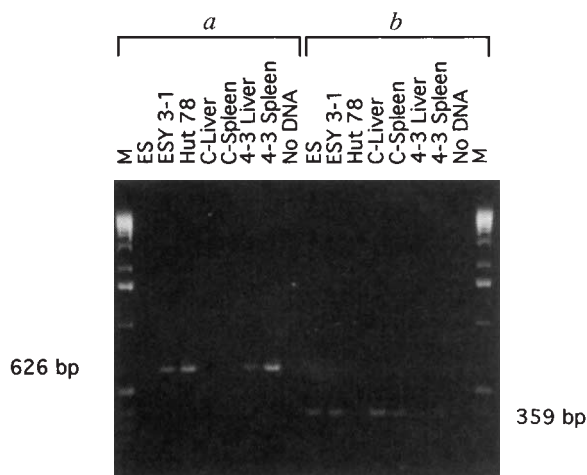


FIG. 4 The human HPRT gene remains functional after transmission through the mouse germ line. *a*, Detection of human HPRT mRNA by reverse transcription (RT)-PCR in ES, ESy 3-1 and Hut 78 (human) cells, spleen and liver from a control mouse (C) or the 4-3 agouti offspring (derived from 394/95-2 chimaera) and a sample containing no template DNA (no DNA). *b*, Detection of mouse γ -interferon receptor mRNA by RT-PCR in the samples above. The specific human HPRT mRNA was also detected in brain, kidney and heart derived from the 4-3 mouse (not shown).

METHODS. Reverse transcription of poly (A)⁺ RNA and PCR amplification of specific cDNA sequences were done using the cDNA Cycle Kit (Invitrogen). Specific amplification of a 626 bp fragment from human HPRT cDNA in the presence of murine HPRT cDNA was done as outlined in ref. 4. Integrity of all RNA samples was demonstrated by PCR amplification of cDNAs for the mouse γ -interferon receptor, using the primers: GTATGTGGAGCATAAC-CGGAG and CAGGTTTGTCTCTAACGTGG to amplify the 359 bp fragment (ref. 22 and M. Arbones, manuscript in preparation). The human HPRT and the γ -interferon receptor primers were designed to eliminate the possibility of obtaining PCR products from genomic DNA contamination. PCR products were analysed by electrophoresis and visualized with ethidium bromide. The size markers (M) are the 1 kb ladder (BRL).

from that of unfused ES cells (Fig. 3A). YAC and yeast DNA sequences were stably retained by the differentiated ESy clones during 40 days of culture in nonselective medium, without impairing pluripotency (Fig. 3B). The differentiated cultures maintained a functional human HPRT gene as shown by their normal growth and differentiation when transferred to HAT-selective medium.

The ability of ESy cells to repopulate mice, including the germ line, was examined by generation of ESy-derived chimaeric mice. Polymerase chain reaction (PCR) analysis specific for the YAC left vector arm demonstrated the presence of vector sequences in tail DNA from all 18 chimaeras derived from 3 different ESy clones and in kidney and spleen DNA from 2 tested mice (not shown). Three chimaeric males, generated from ESy 3-1, a clone containing a complete *Alu* profile, left vector arm, complete *Ty* profile, but not the right YAC arm (Fig. 1), transmitted the ES cell genome to their offspring at a frequency of 30%. In 3 of 12 agouti offspring, all yHPRT and yeast sequences present in the parent ESy clone 3-1 were detected (Fig. 3C), demonstrating faithful transmission of the integrated fragments. Using a human HPRT-specific PCR assay on messenger RNA-derived complementary DNA from a yHPRT-containing offspring, we detected the expression of the human HPRT gene in spleen, liver (Fig. 4), brain, heart and kidney (not shown), demonstrating that the transmitted YAC DNA retained its function with fidelity. Our results indicate that the uptake of megabases of yeast genomic DNA was not detrimental to proper development, germ-line transmission or gene expression.

Our findings open the way for the use of megabase-sized loci for genetic complementation studies of recessive genetic disorders as well as for the generation of novel mouse strains: mice

containing the human germ-line immunoglobulin gene array, capable of producing a full repertoire of human antibodies, or mice that serve as models for human diseases such as those involving partial chromosome trisomy (for example Down's syndrome).

Note added in proof: Comparable steady-state levels of mouse and human HPRT mRNA were detected in the liver of yHPRT-containing offspring. □

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A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice

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EXPRESSION of transgenes in mice often fails to follow the normal temporal and spatial pattern and to reach the same level as the endogenous copies^{1,2}. Only in exceptional cases has position-independent and copy number-dependent expression been reproduced³. The size constraint of standard constructs may prevent the inclusion of important remote regulatory elements. Yeast artificial chromosomes (YACs)⁴ provide a means of cloning large DNA fragments and the transfer of YAC DNA into somatic cells has been reported⁵⁻⁹. We have previously produced transgenic mice carrying a 35 kilobase YAC construct¹⁰. Here we report the transfer of a 250 kilobase YAC covering the mouse tyrosinase

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gene into mice by pronuclear injection of gel-purified YAC DNA. The YAC was inserted into the mouse genome without major rearrangements and expression of the YAC-borne tyrosinase gene resulted in complete rescue of the albino phenotype of the recipient mice. Expression from the transgene reached levels comparable to that of the endogenous gene and showed copy number dependence and position independence.

A yeast artificial chromosome covering the mouse tyrosinase locus was isolated from a YAC library of C3H mouse DNA¹¹. The YAC was modified in yeast by homologous recombination to introduce a conditional centromere for amplification^{10,12} in yeast (A.S. *et al.*, manuscript in preparation). YRT2, the resulting 250 kilobase (kb) construct, contains 80 kb of the tyrosinase coding region¹³, 155 kb of upstream sequences and 15 kb of vector DNA (Fig. 1). We devised a purification protocol to obtain high quality DNA at a concentration suitable for microinjections (see legend to Table 1). Pronuclear injections of YRT2 DNA into fertilized oocytes of albino NMRI mice were done using standard methods¹⁴. The albino phenotype allowed mice expressing the transgene to be identified at birth by the presence of tyrosinase activity and hence pigment in the eye and skin.

Out of 24 newborns, 5 (21%) showed pigmentation (Table 1). This efficiency of transgenesis is comparable to that obtained with much smaller constructs^{1,15}. At weaning, DNA from all mice was tested for the presence of YAC vector sequences using probes from the long and short arms¹⁰. Only the five pigmented mice were positive, four of which showed hybridization to probes from both vector arms (Table 1). One pigmented mouse died at birth, a second one was found to be sterile, whereas a third (number 1971) was a mosaic as judged by coat colour and transmission analysis. The two remaining nonmosaic founders (numbers 1976 and 1999) transmitted the transgene and hence the pigmentation as a mendelian trait. More detailed Southern analysis was done on F₁ offspring obtained by mating the transmitting NMRI founders (numbers 1971, 1976 and 1999) with BALB/c mice (Fig. 2a). Hybridization with probes for the short (Fig. 2a, probe a) and long (Fig. 2a, probes b and c) vector arm

TABLE 1 Pronuclear injection of YRT2 DNA allows generation of transgenic mice at high frequency

Oocytes injected	Mice born	Transgenic mice	Pigmented mice
393	24	5	5

YAC DNA was isolated as described below and injected into fertilized NMRI oocytes¹⁴ using DNA preparations at a concentration of $\sim 4 \text{ ng } \mu\text{l}^{-1}$. This table combines the results of experiments with two independent batches of DNA. The 393 injected oocytes were transferred to 12 pseudopregnant NMRI females, of which 7 (which received 240 oocytes) became pregnant. Transgenic mice were identified by preparing DNA from tail biopsies and simultaneous hybridization with the 4.5 kb and 5.4 kb *Hind*III fragments of YAC vector Y-RC16 (ref. 10). Expressing mice were recognized by the presence of pigment in the eye and skin. METHODS. The YAC containing yeast strain was grown under amplification conditions^{10,12} and DNA in agarose blocks prepared according to ref. 25. DNA was separated on a 1% low melting point (LMP)-agarose (FMC, Seaplaque GTG) pulsed field gel electrophoresis (PFGE) gel (LKB-Pulsaphor) in 0.25×Tris-acetate/EDTA buffer (TAE, 1×TAE=40 mM Tris-acetate, 1 mM EDTA) at 250 V with the following pulses: t₁=9 s, 10 h, t₂=15 s, 6 h. The YAC-containing gel slice was excised after staining marker lanes on either side of the gel. After equilibration in 1×TAE the slice was positioned on a minigel tray and embedded in 4% Nusieve GTG agarose (FMC). A second gel run was done at a 90° angle to the PFGE run for 3 h at 4 V cm⁻¹ resulting in a roughly fourfold concentration of the YAC DNA at the border to the high percentage agarose. The excised gel slice containing YAC DNA was equilibrated in 1×TAE, 100 mM NaCl, 30 μM spermine, 70 μM spermidine and digested for 2 h with 4 U Gelase (Epicentre) per 100 mg of gel slice. Finally the DNA was dialysed for 2 h on a floating dialysis filter (Millipore, pore size 0.05 μm) against injection buffer (10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 100 mM NaCl, 30 μM spermine and 70 μM spermidine). YAC DNA was routinely obtained at a concentration of 4 to 10 $\text{ng } \mu\text{l}^{-1}$ and used directly for injections.

revealed that both ends are retained in the three transgenic lines. Probe b (Fig. 2a) detected bands due to apparently rearranged DNA in the multicopy line number 1971, in addition to that of the expected size. A vector fragment located closer to the insert hybridized to a fragment of the expected size (Fig. 2a, probe c), which suggests that the rearrangements seen with probe b are restricted to the end of the long arm of the vector and may have arisen from exonuclease digestion before integration. To determine the exact number of copies of the YAC transgene integrated in each of the lines, we used an *Eco*RI restriction fragment length polymorphism (RFLP) between the endogenous and YAC-derived tyrosinase gene detected with probe d (Fig. 2a)¹⁶. Quantification revealed the presence of one (number 1999), two (number 1976) and eight (number 1971) copies of the transgene. The same ratio (1:2:8) was seen with probes a and c (Fig. 2a). The identical copy number for an internal fragment and both vector arms was considered as a strong indication that the YAC transgene had been integrated intact.

Rescue of the albino phenotype by expression of a tyrosinase minigene in transgenic mice has been described previously^{15,17,18}, but pigmentation of these transgenic lines was variable resulting from widely varying tyrosinase expression. To allow a better comparison of the coat colour between our lines, founders number 1971, 1976 and 1999 were mated with BALB/c mice (genotype: *AA bb cc*). The outbred strain NMRI is not defined for the coat colour loci brown (*b*) and agouti (*a*), which act downstream of the tyrosinase locus (*c*) in determining the quality of pigmentation. The F₁ offspring of the three YAC transgenic lines had a homogeneous strong agouti coat colour comparable to the pigmentation of wild-type mice with no obvious differences between lines (Fig. 2b).

To investigate the level of expression of the transgenes in skin melanocytes, we analysed skin RNA from the F₁ offspring by reverse transcriptase polymerase chain reaction (RT-PCR) (Fig. 3). NMRI and BALB/c mice are albino because they carry a point mutation in the first exon of the tyrosinase gene leading

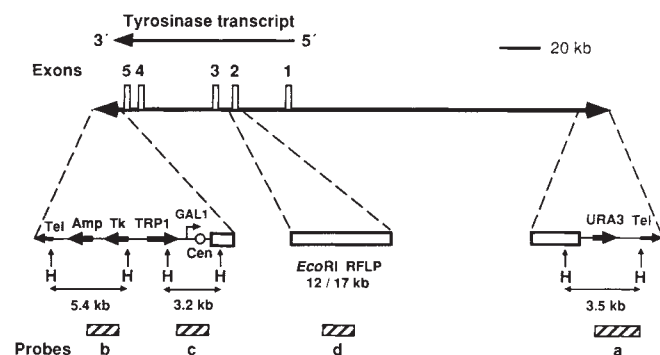


FIG. 1 Characteristics of the yeast artificial chromosome YRT2 containing the mouse tyrosinase gene. The length of the YAC is 250 kb. YRT2 is a deletion derivative of a YAC from a C3H YAC library¹¹. The long arm from Y-RC16 (ref. 10), which allows amplification of the YAC, was inserted 3' to the tyrosinase gene by homologous recombination in yeast (A.S. *et al.*, manuscript in preparation). The short arm is retained from the original isolate from the library. Below, the vector arms and regions relevant to the Southern analysis shown in Fig. 2a are expanded. Probes are depicted as hatched boxes. *Hind*III sites defining the fragments detected by probes a, b and c are indicated as H. Numbers 1–5 indicate the exons of the tyrosinase gene¹³. An *Eco*RI RFLP¹⁶ in exon number 2 detected by probe d allows the transgenic tyrosinase allele (17 kb) to be distinguished from endogenous NMRI and BALB/c alleles (12 kb) (Fig. 2). Markers and functional elements from the vector arms are given as filled arrows (except the open circle, which indicates the centromere) with the following abbreviations: Tel, telomeres; Amp, ampicillin resistance gene; Tk, thymidine kinase gene of herpes simplex virus; Cen, centromere (*CEN4*) associated with the *GAL1*-promotor (*GAL1*); *TRP1* and *URA3*, yeast markers according to ref. 12.

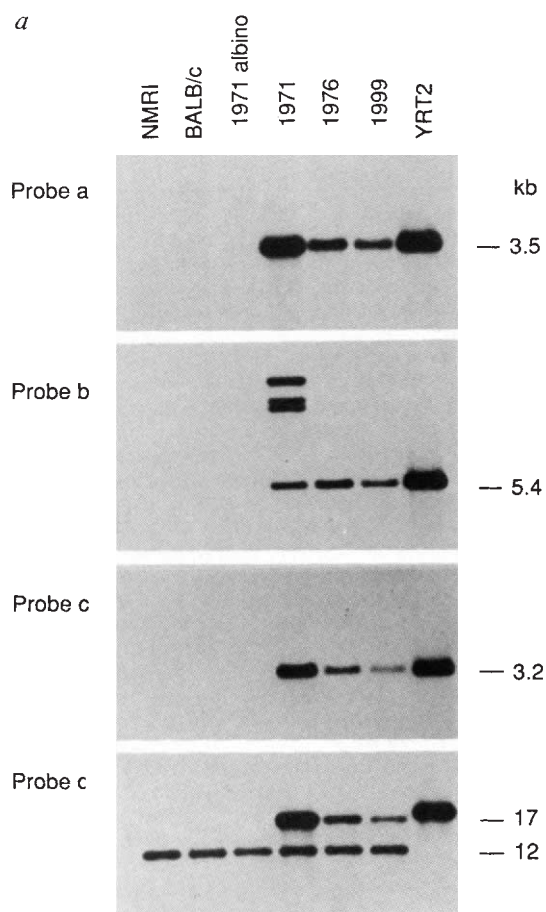
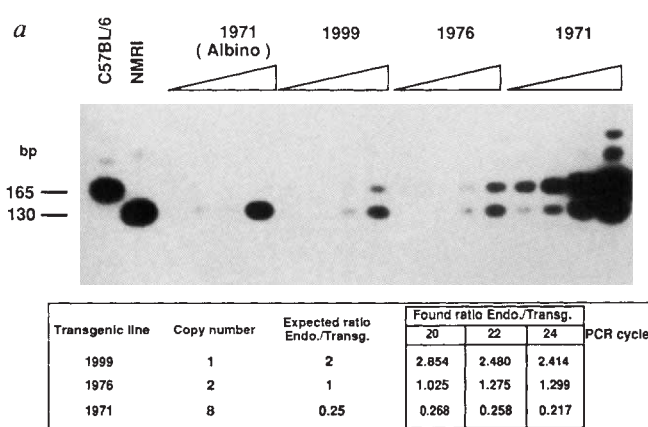


FIG. 2 *a*, Southern analysis of F_1 offspring from transgenic lines number 1971, 1976 and 1999 reveals integration of the YAC DNA. DNA from NMRI and BALB/c mice and a nontransgenic littermate of line number 1971 (1971 albino) as well as ~ 2 ng of isolated YRT2 DNA was included as negative and positive controls, respectively. F_1 mice from all three transgenic lines are positive for both vector arms (probes a to c, Fig. 1). Rearranged fragments seen with probe b in line number 1971 are no longer visible with probe c. The lowest panel shows determination of copy number of the tyrosinase transgene using an *EcoRI* RFLP (probe d, Fig. 1; ref. 16). The 12 kb fragment represents the endogenous gene (two copies) and was used as an internal standard. *b*, Complete rescue of the albino phenotype in transgenic animals. F_1 offspring of lines number 1971, 1976 and 1999 and a nontransgenic littermate (1971 albino) are shown. No difference in coat colour was observed between mice from the three transgenic lines. METHODS. *a*, Transgenic founders were crossed with BALB/c mice and DNA was prepared from kidneys²⁶. DNA (5 μ g) was digested with *HindIII* for probes a to c (the same filter was used for the three probes), and *EcoRI* for probe d. Gel electrophoresis, blotting and hybridization was done as described before²⁷. Radioactivity accumulated in the bands was quantified with a Phosphorimager (Molecular Dynamics).

FIG. 3 Analysis of the tyrosinase messenger RNA expression from the YAC transgene reveals copy number dependence. *a*, RT-PCR analysis of F_1 offspring from the three transgenic lines (number 1971, 1976 and 1999) and a nontransgenic littermate of line 1971 (1971 albino). Tyrosinase mRNA sequences corresponding to exon 1 were amplified by RT-PCR with oligonucleotides A and B (see c). Multiple reactions were set up in parallel and stopped after 18, 20, 22 and 24 cycles (indicated by triangles) within the logarithmic phase of amplification. PCR products were digested with *DdeI* and detected by hybridization with oligonucleotide C (see c) to reveal the polymorphism between products from the endogenous (albino) and YAC tyrosinase transcripts. The 130 bp product in the F_1 offspring derives from the albino alleles of NMRI and BALB/c and the 165 bp product from the transgene. Differences in the absolute amounts of products between lines might reflect a variable contribution of melanocytes in tissue sample taken. Lanes C57BL/6 and NMRI are PCR products from genomic DNA used as controls for the *DdeI* RFLP. The table below shows the quantification of cycles 20, 22 and 24 expressed as a ratio between the endogenous and transgenic products. *b*, Analysis of mixtures of skin RNA from C57BL/6 and NMRI mice at the indicated ratios provided a control for the quantification of the RT-PCR. *c*, Scheme for the PCR analysis indicating the use of the polymorphic *DdeI* site (shown in brackets) defined by the albino mutation to discriminate the products of the transgenic and wild-type alleles (YRT2 and C57BL/6, 165 bp) from the endogenous mutant allele (NMRI and BALB/c, 130 bp). Position of oligonucleotides A, B and C is indicated. Oligonucleotides A and B are designed according to ref 20.

METHODS. First strand complementary DNAs were generated by incubating total skin RNA¹³ according to a protocol adapted from refs 28, 29 and 30. RNAs (1 μ g) were heated to 70 °C for 5 min, cooled on ice, and then incubated at 37 °C for 1 h in a final volume of 20 μ l with 1 μ g of random hexamer primers (Pharmacia), 20 U of MMLV reverse transcriptase (Boehringer-Mannheim), 200 μ M of each dNTP (Pharmacia) and 4 U of RNasin (Promega) in a RT-buffer containing 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM DTT and 3 mM Mg_2Cl_2 . cDNA products were purified by ethanol precipitation and



resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). 1/10 of the product was used for further PCR analysis combined with 100 pmols of oligonucleotide A (5'-TTCAAAGGGTGGATGACCG-3', bases 293-312, numbered according to ref. 31) and 100 pmol oligonucleotide B (5'-GGATG-CATTACTATGTGTCA-3', bases 634-615 (ref. 31)), 2.5 U of *Taq* polymerase (Beckman), 200 μ M of each dNTP, and the following PCR buffer (50 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM DTT, 2 mM Mg_2Cl_2) in a total volume of 50 μ l. Paraffin oil (50 μ l) was layered on top of each tube. Each PCR cycle was 30 s at 93 °C, 30 s at 55 °C and 90 s at 72 °C in a Perkin Elmer Thermocycler. PCR products were phenol extracted, ethanol precipitated and digested with *DdeI*. After *DdeI* digestion, 1/10 of the products were separated in a 4% NuSieve GTG Agarose (FMC) in 1 $\times$ TAE at 4 V cm^{-1} for 4 h and then blotted onto nylon membranes (Biohyde). Oligonucleotide C (5'-

to the production of an inactive protein^{17,19}. We used a *DdeI* RFLP determined by this point mutation²⁰ to compare the level of expression of the transgenes with respect to the endogenous gene. Quantification showed that the level of expression per copy of transgene was comparable to that of the endogenous gene (Fig. 3), which indicated copy number dependence and position independence. RT-PCR analysis of additional tissues (data not shown) revealed that the expression of the transgenes had the same tissue-specific pattern as the endogenous gene¹⁵. The faithful expression of this large and complex transcription unit also argues strongly for integration of unrearranged YAC DNA.

The complete rescue of the albino phenotype of the YRT2 transgenic mice in contrast to mice carrying tyrosinase minigenes^{15,17,18}, suggests an effect of position and/or the absence of elements necessary for correct expression in the minigene constructs. This could include sequences with properties of dominant control regions, such as those found in the β -globin gene cluster³ and lysozyme gene²¹. Alternatively, the position-independent and copy number-dependent expression might be due to elements present in the 250 kb construct mediating boundary effects, analogous to those identified in the *Drosophila* genome²². One important element might be the DNase I hypersensitive site -15 kb upstream of the tyrosinase gene²³, which is present in B16 mouse melanoma cells but not in mouse NIH3T3 fibroblasts (R. Ganss and G.S., unpublished data). Analysis of deletion derivatives of YRT2 will help to identify the sequences responsible for the correct expression of the tyrosinase gene.

We envisage that YACs covering a locus and sufficient flanking sequences will be of great use for transgenic experiments where correct expression is essential. This will benefit the analysis of large gene clusters in their natural context, such as the globin and the Hox genes, and of far upstream regulatory elements. It will assist the identification of genes by complementation and investigation of long-range regulatory

phenomena such as X-inactivation and imprinting. Such analyses will be aided by the ease with which defined mutations and markers can be introduced into YAC constructs by homologous recombination in yeast²⁴. □

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MAP kinase-related FUS3 from *S. cerevisiae* is activated by STE7 *in vitro*

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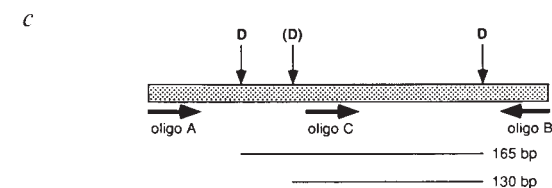
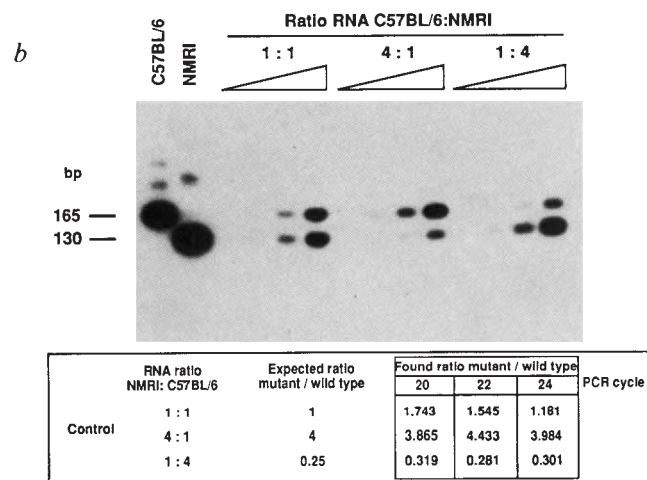
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PHEROMONE-STIMULATED haploid yeast cells undergo a differentiation process that allows them to mate¹. Transmission of the intracellular signal involves threonine and tyrosine phosphorylation of the redundant FUS3 and KSS1 kinases, which are members of the MAP kinase family²⁻⁴. FUS3/KSS1 phosphorylation depends on two additional kinases, STE11 and STE7 (refs 2, 5, 6). Genetic analyses predict an ordered pathway where STE11 acts before STE7 and FUS3/KSS1 (refs 2, 7). Here we report that STE7 is a dual-specificity kinase that modifies FUS3 at the appropriate sites and stimulates its catalytic activity *in vitro*. From these data and previous genetic results, we argue that STE7 is the physiological activator of FUS3. Recent indications that MAP kinase activators are related to STE7 suggest that signal transduction pathways in many, if not all, eukaryotic organisms use homologous kinase cascades⁸⁻¹⁰.

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GGATTGGGGGCCCAATTGTACAGAGAAGCGAGTCTGA-3', bases 398-437 (ref. 31)) was labelled with [γ -³²P]ATP and polynucleotide kinase (NEB) for hybridization and detection of the *DdeI* RFLP. Quantification of the bands was made by a Phosphorimager (Molecular Dynamics).

To study FUS3 phosphorylation *in vitro* we isolated it as a Myc epitope-tagged fusion protein from *Escherichia coli* (Fig. 1a). We expressed and isolated two inactive mutant derivatives of FUS3 in a similar way. One has a substitution at the conserved catalytic lysine (FUS3M-R42) and the other has two additional changes at the major phosphorylation sites (FUS3M-R42A180F182). The wild-type FUS3M incorporated radioactive phosphate in autophosphorylation assays but the FUS3M-R42 derivative was inactive (Fig. 1b). The autophosphorylation product was different from the *in vivo* phosphorylated FUS3, because the former was modified on serine and tyrosine but not on threonine (Fig. 1c).

To see if the STE7 kinase could phosphorylate FUS3, we isolated a Myc epitope-tagged version of STE7 (STE7M) from yeast extracts by immune affinity purification. To eliminate FUS3M autophosphorylation, we used the catalytically inactive FUS3M-R42 protein from *E. coli* as the substrate. Phosphorylation of the FUS3M-R42 protein occurred in the reactions with the active STE7M but not the catalytic lysine substitution mutant, STE7M-R220 (Fig. 2a, lanes 1, 2, 4 and 5). There was more FUS3M-R42 phosphorylation when STE7M was isolated from pheromone-induced cells (Fig. 2a, lanes 1 and 4). This increase must be attributed to a more active STE7 kinase, because the amount of STE7M was the same in extracts from pheromone-induced and uninduced cells (data not shown). As mentioned, STE11 is a second kinase whose function is necessary for FUS3 modification *in vivo*². Under our assay conditions, immune affinity-purified STE11M kinase did not phosphorylate FUS3M-R42 (data not shown). This provides additional evidence that phosphorylation of FUS3M-R42 is specifically catalysed by STE7M.

A second protein was phosphorylated in the STE7M immune complex kinase assays (Fig. 2). This protein was STE7M, because an N-terminally truncated but still biologically active form of the STE7M kinase (STE7M-ΔS) caused a shift in the position of the signal (Fig. 2a, lanes 3 and 6). The absence of other phosphoproteins suggests that no coprecipitating kinase intervenes between STE7 and FUS3.

Phosphorylation of FUS3M-R42 in the assays with STE7M occurred on serine, threonine and tyrosine residues (Fig. 2b).

Most of the modification must be on the threonine 180 and tyrosine 182 sites of *in vivo* phosphorylation because there was much less ³²P incorporation with the FUS3M-R42A180F182 derivative (Fig. 2c). We propose therefore that STE7 is a dual specificity kinase sufficient for the threonine and tyrosine phosphorylation of FUS3 (ref. 11).

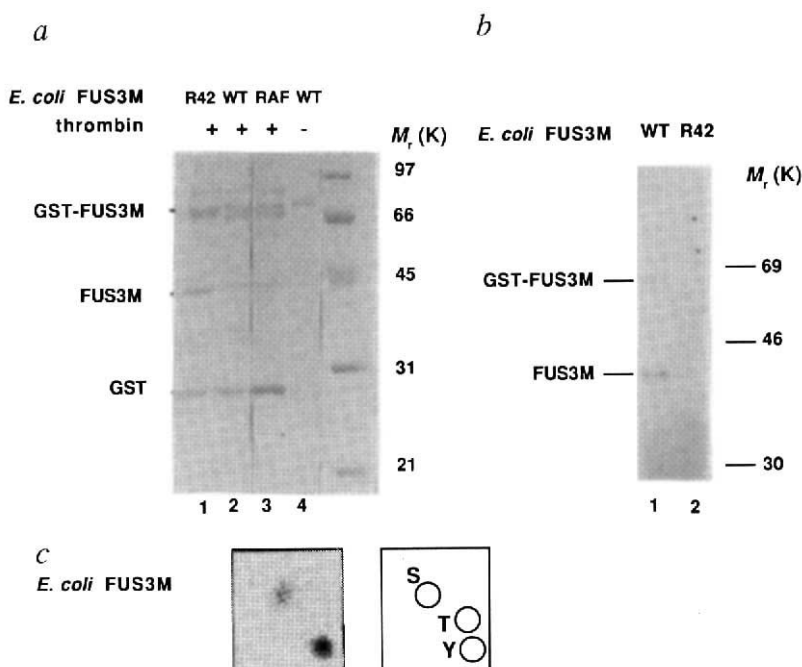
The effect of pheromone induction on FUS3 kinase activity was investigated using an immune complex assay with a Myc epitope-tagged FUS3 (FUS3M). Myelin basic protein (MBP), a standard substrate for vertebrate MAP kinases, was phosphorylated when FUS3M was immune affinity purified from extracts of induced but not uninduced cells (Fig. 3, lanes 1 and 2). Two lines of evidence show that this stimulation of kinase activity is due to pheromone-induced FUS3 phosphorylation. First, the FUS3M-A180F182 mutant, which has substitutions at both *in vivo* sites of modification, did not phosphorylate MBP (Fig. 3, lanes 5 and 6). Second, treatment of FUS3M with phosphatase 2A abolished phosphorylation of the FUS3-specific substrate (data not shown).

FUS3M was also phosphorylated in the immune complexes prepared from pheromone-induced cells. This cannot be due to autophosphorylation because the signal was actually stronger in immune complexes with the inactive FUS3M-R42 protein (Fig. 3, lanes 3 and 4). Therefore, a second kinase was coprecipitated with FUS3M. We think that this kinase is STE7 because a phosphoprotein with the same mobility as STE7 was observed in the reaction with pheromone-induced FUS3M (Fig. 3, lane 2; see below). Additionally, FUS3M-R42 was not phosphorylated when immune complexes were from extracts of pheromone-induced *ste7Δ* mutant cells (data not shown). Note that FUS3M-R42 was phosphorylated to a considerable extent in extracts from uninduced cells (Fig. 3, lane 4), consistent with previously published *in vivo* data suggesting that the basal level of signal activity is actually higher in cells without functional FUS3 (ref. 2).

FUS3 kinase activity can also be stimulated through its *in vitro* modification by STE7M. For these activation assays we used an N-terminal fragment of the FAR1 protein as a FUS3-specific substrate¹². This polypeptide is a more sensitive and specific substrate than MBP (M. Peter, A.G., G.A. and

FIG. 1 Purification of FUS3 kinase from *E. coli* and its autocatalytic properties. **a**, Coomassie blue stained gel of SDS-PAGE-fractionated GST fusion proteins purified from *E. coli* after (lanes 1–3) and before (lane 4) cleavage with thrombin. Samples of GST-FUS3M (wt), GST-FUS3M-R42 (R42), and GST-FUS3M-R42A180F182 (RAF) preparations are shown. Position of the GST-FUS3M fusion protein, the cleaved FUS3M protein and the GST polypeptide are indicated. **b**, Autophosphorylation assays with FUS3M (wt) and FUS3M-R42 (R42). **c**, Phosphorimager print of [³²P]phospho-amino acids from FUS3M after autophosphorylation (left). Position of phospho-serine (S), -threonine (T) and -tyrosine (Y) standards (right).

METHODS. FUS3M, FUS3M-R42 and FUS3M-R42A180F182 proteins were expressed as GST fusion proteins in a BL21 (DE3) *E. coli* strain using plasmids pGA1965, pGA1944 and pGA1945, respectively. To construct these expression plasmids, the *Nde*I-*Bam*HI fragments encoding tagged FUS3 (FUS3M) and derivatives were isolated from pGA1903, pGA1905 and pGA1932 (ref. 20) and cloned into plasmid pGEX-2T6 (provided by G. Draetta). Except for the addition of an *Nde*I site, pGEX-2T6 is the same as pGEX-2T (ref. 14). Fusion protein synthesis was induced at room temperature and the proteins were purified as described¹⁴. Autophosphorylation assays were done in a final reaction volume of 20 μl that included 250 ng of purified FUS3M or FUS3M-R42 and 10 μCi [³²P]ATP. Kinase assay buffer was as described¹⁵ but β-glycerol phosphate was omitted. After 30 min incubation at 30 °C, reactions were stopped by addition of SDS-PAGE sample buffer¹⁶ and boiling for 3 min. Phosphoproteins were fractionated



by SDS-PAGE¹⁶ and visualized by autoradiography of the dried gel. The analysis of FUS3M phospho-amino acids was as previously described².

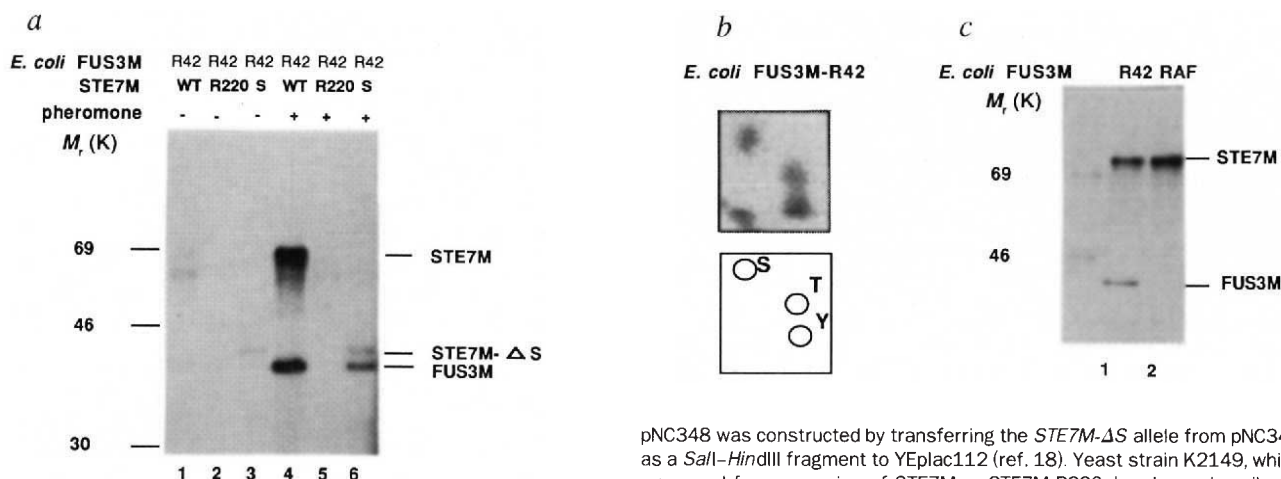


FIG. 2 STE7 phosphorylates FUS3. **a**, Comparison of STE7 kinase activity from uninduced (–) and induced (+) cultures. Immune affinity-purified STE7M (wt), STE7M-R220 (R220) or STE7M-ΔS (S) was present as indicated. Each assay mixture contained 250 ng purified FUS3M-R42 (R42) as substrate. Note that STE7M from pheromone-induced cells is hyperphosphorylated causing it to have a lower mobility than when isolated from uninduced cells (lanes 1 and 4). **b**, Phosphorimager print of [32 P]phospho-amino acids in FUS3M-R42 from **a**, lane 4 (top). Migration of phospho-serine (S), -threonine (T) and -tyrosine (Y) standards (bottom). **c**, Comparison of FUS3M-R42 (R42, 250 ng) and FUS3M-R42A180F182 (RAF, 250 ng) as substrates for immune affinity-purified STE7M (wt) from pheromone-induced cells.

METHODS. The plasmids pNC267 and pNC267-R220 for expression of STE7M and STE7M-R220 will be described elsewhere²⁰. The STE7M-ΔS allele was constructed by site-directed mutagenesis using the STE7 phagemid pNC318 as source of template DNA and oligonucleotide 5'-CGTATCTTTCAGATCTGCG-CCTTCTATTCG as the mutagenic primer¹⁷. The resulting plasmid (pNC346) has a deletion in STE7 that encompasses codons 45–170. The plasmid

pNC348 was constructed by transferring the STE7M-ΔS allele from pNC346 as a *Sall*–*HindIII* fragment to YEplac112 (ref. 18). Yeast strain K2149, which was used for expression of STE7M or STE7M-R220, has been described². Yeast strain E929-6C-35 (STE7Δ FUS3 KSS1 bar1Δ), which was used for expression of STE7M-ΔS, is isogenic to strain E929-6C (ref. 5). Where pheromone induction is indicated, cultures were exposed to 50 nM α -factor for 1.5 h. Extracts were prepared as described¹⁵ but with modified stop and lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 (NP-40), 50 mM NaF, 30 mM Na₂H₂P₂O₇, 15 mM 4-nitrophenylphosphate, 0.1 mM ortho-vanadate, 1 mM PMSF, 40 μ g ml^{–1} aprotinin and 20 μ g ml^{–1} leupeptin). For immune affinity purification of STE7M or derivatives, 100 μ g of the specified extract was incubated with 4 μ g of Myc1-9E10 antibodies for 1 h on ice¹⁹. The antibody–antigen complexes were incubated with protein A sepharose beads (10 μ l packed volume) at 4 °C for 1.5 h. The adsorbed immune complexes were washed five times in buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 15 mM 4-nitrophenylphosphate, 0.1 mM ortho-vanadate), three times in buffer B (25 mM MOPS pH 7.2, 15 mM 4-nitrophenylphosphate, 0.1 mM ortho-vanadate) and once in buffer B plus 15 mM MgCl₂. Substrate phosphorylation assays with the immune complexes and analysis of phosphorylation products were as described in Fig. 1, except that 2 μ Ci of [γ -³²P]ATP was used per reaction.

I. Herskowitz, manuscript in preparation). Addition of purified FUS3M and FAR1 proteins to STE7M immune complexes from extracts of pheromone-induced *kss1 fus3* double mutant cells resulted in FAR1 phosphorylation (Fig. 4a, lane 3). No modification of FAR1 occurred without the addition of FUS3M (Fig. 4a, lanes 1 and 2). When FUS3M was present with the inactive STE7M-R220, we observed much less FAR1 phosphorylation (Fig. 4a, lane 4). We suppose that the autophosphorylated form of FUS3 could be responsible for this minor activity.

STE7 is also a substrate of the FUS3 kinase. STE7M was phosphorylated in reactions where the wild-type FUS3M has been activated (Fig. 4b, lane 3) but not in reactions with the inactive FUS3M-R42 (Fig. 4b, lanes 1 and 2). This is consistent with the observation that hyperphosphorylation of STE7 *in vivo* depends on an active FUS3 or KSS1 kinase⁷. The role of this reciprocal phosphorylation of STE7 by FUS3 is unclear but it

could be part of an attenuation mechanism. This proposal is consistent with a higher signal activity in *fus3 kss1* double mutant cells compared with wild-type cells as indicated by the relative amounts of FUS3-R42 phosphorylation (ref. 2 and Fig. 3, lane 4). Additionally, STE7M kinase catalysed more FUS3-R42 phosphorylation when it was isolated from a *fus3 kss1* strain compared with a wild-type strain (Fig. 4c). Because the same amount of STE7 was present in the two extracts (data not shown), STE7 seems to be more active when isolated from cells without FUS3 or KSS1.

It has been noted that peptide sequences obtained from purified *Xenopus* and rabbit skeletal muscle MAP kinase activators show similarities to the STE7 sequence^{8–10}. The case for a close relationship between MAP kinase kinase and STE7 has been made even stronger by recent results²¹. This suggests that the kinase cascade we have characterized in yeast will be

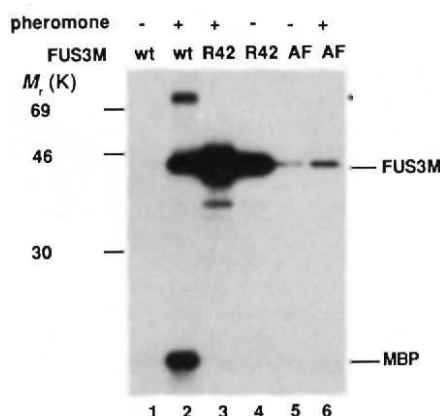


FIG. 3 Pheromone potentiates FUS3 kinase activity. Results of phosphorylation assays with 10 μ g of bovine myelin basic protein (MBP) added to immune affinity-purified FUS3M (wt), FUS3M-R42 (R42) and FUS3M-T180Y182 (AF) from uninduced (–) or pheromone-induced (+) cultures. The positions of FUS3M and MBP are indicated. The asterisk marks one co-immune-precipitated protein that is only phosphorylated in reactions with pheromone-induced and catalytically active FUS3M.

METHODS. FUS3M (pGA1903), FUS3M-R42 (pGA1905) and FUS3M-A180F182 (pGA1906) were expressed in yeast strain K2314 (STE7 *fus3Δ kss1Δ bar1Δ*)². Where pheromone induction is indicated, cultures were exposed to 50 nM α -factor for 1 h. Extract preparation, immune affinity purification of FUS3M (or derivatives) and phosphorylation assays were essentially as described in Fig. 2.

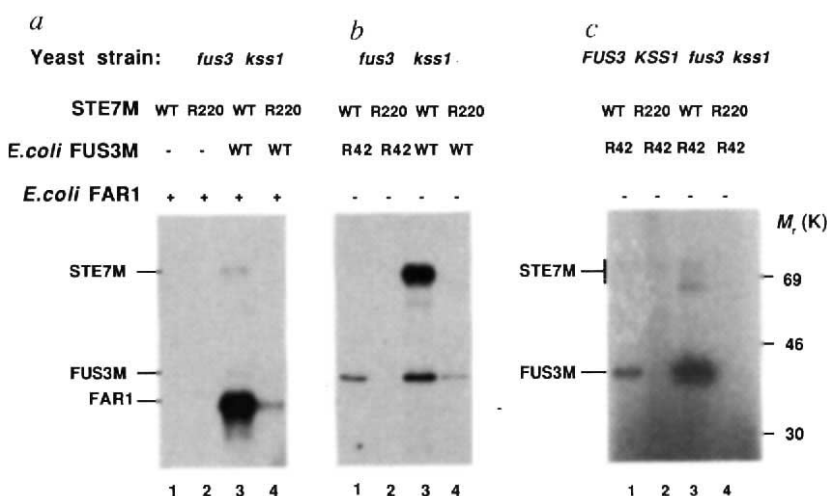
FIG. 4 STE7 reconstitutes activation of the FUS3 kinase. **a**, Phosphorylation assays showing STE7 stimulates FUS3-dependent phosphorylation of FAR1. STE7M (wt, pNC318) or STE7M-R220 (R220, pNC318-R220) was immune affinity purified from pheromone-treated cultures of K2314 (*fus3 kss1*). FUS3M (250 ng) produced and purified from *E. coli* was either present (wt) or absent (–) from the reactions. The target substrate, an N-terminal fragment of FAR1 (250 ng) was present (+) in all assays. The positions of phosphorylated FAR1 fragment, FUS3M and STE7M are indicated. **b**, Phosphorylation assays showing that activated FUS3 phosphorylates STE7. Purified FUS3M (wt; 250 ng) or FUS3M-R42 (R42; 250 ng) was added to STE7M (wt) and STE7M-R220 (R220) isolated as specified in **a**. **c**, Comparison of STE7M kinase activity after isolation from *FUS3 KSS1* or *fus3 kss1* genetic backgrounds. Phosphorylation assays with 250 ng of purified FUS3M-R42 as substrate and immune affinity-purified STE7M (wt) or STE7M-R220 (R220) from pheromone-induced cultures of strain K2180 (lanes 1 and 2) and strain K2314 (lanes 3 and 4).

METHODS. The plasmids pNC318 and pNC318-R220 for expression of STE7M and STE7M-R220, respectively, will be described elsewhere²⁰. Yeast strains K2314 (*STE7 fus3Δ kss1Δ bar1Δ*) and K2180 (*STE7 FUS3 KSS1 far1Δ bar1Δ*) are isogenic with strain K2149 (ref. 2).

found in many if not all eukaryotic cells. In yeast, the cascade consists of the STE11, STE7 and FUS3/KSS1 kinases whose equivalents in mammals may be the Raf kinase (or perhaps another kinase equivalent to STE11), a STE7-like MAP kinase activator and MAP kinase^{8–10,13}. □

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Expression and purification of the N-terminal FAR1 fragment (encompassing amino acids 1–268) used as substrate will be described elsewhere (M. Peter *et al.*, manuscript in preparation). Phosphorylation assays were as described in Fig. 2.

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Pituitary hormone FSH directs the CREM functional switch during spermatogenesis

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THE CREM (cyclic AMP-responsive element modulator) gene encodes multiple regulators of the cAMP-transcriptional response by alternative splicing¹. A developmental switch in CREM expression occurs during spermatogenesis, whereby CREM function is converted from an antagonist to an activator (CREM τ ; ref. 2) which accumulates to extremely high levels from the pre-meiotic spermatocyte stage onwards. To define the physiological mechanisms controlling the CREM developmental switch, we have hypophysectomized rats and observed the extinction of CREM τ expression in testis, thereby demonstrating a central role of the pituitary–hypothalamic axis. We then used the seasonal-dependent

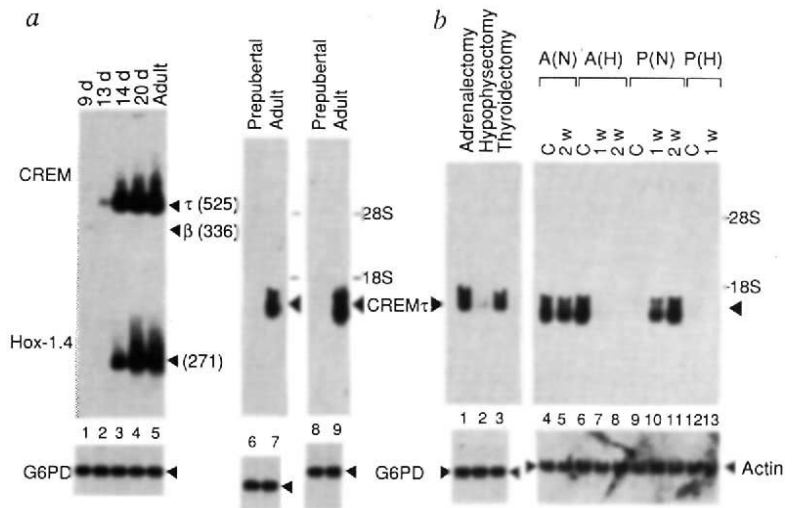
modulation of spermatogenesis in hamsters to dissect the hormonal programme controlling this developmental process. By this approach, combined with direct administration of pituitary-derived hormones, we have established that follicle-stimulating hormone (FSH) is responsible for the CREM switch. FSH appears to regulate CREM expression by alternative polyadenylation, which results in a dramatic enhancement of transcript stability.

We have shown that the amount of the CREM transcript increases dramatically from the pachytene spermatocyte stage onwards and encodes the activator CREM τ instead of the antagonists². In mouse the switch occurs between days 13 and 14 (Fig. 1a, lanes 1–5). This functional switch is conserved in other species at an equivalent stage during spermatogenesis (hamster and rat, see lanes 6–9). As testicular functions are under the control of the pituitary–hypothalamic axis^{3,4}, we tested whether surgical removal of the pituitary gland could affect CREM expression. CREM τ expression is dramatically reduced in the adult rat one week after surgery (Fig. 1b, lanes 2, 7, 8). Removal of other endocrine glands has no detectable effect (lanes 1 and 3). To test whether there is a pituitary control of the developmental switch of CREM in testis, we hypophysectomized prepubertal rats. No equivalent switch in CREM expression or transcript accumulation was detected during subsequent development (Fig. 1b, lanes 9–11 and 12, 13). Thus a functional pituitary is necessary for the switch and maintenance of CREM transcript abundance and splicing. In addition, we analysed *Hox-1.4* expression, which is also developmentally regulated during spermatogenesis⁵. *Hox-1.4* induction is almost coincidental with that of CREM τ (Fig. 1a). *Hox-1.4* expression was

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FIG. 1 a, The switch in CREM expression coincides with *Hox-1.4* induction. Lanes 1–5: polymerase chain reaction (PCR) analysis of testis RNA (9, 13, 14, 20 days old and adult mice). Reactions using CREM- and *Hox-1.4*-specific primers were analysed by Southern blotting. *Hox-1.4* and the CREM τ and β -isoforms are indicated. Sizes of PCR-generated fragments are indicated in parentheses. The CREM switch slightly precedes the appearance of the *Hox-1.4* transcript. Lanes 6–9: northern analysis of 10 μ g total testis RNA. In both rat and hamster, CREM τ is an abundant transcript in adult testis (lanes 7, 9), but it is not detectable in prepubertal (20–23 days old) testis (lanes 6, 8). **b**, The pituitary is required for the CREM switch. Northern analysis of RNA from rat testis after surgery: 'C' indicates normal or surgically treated animals killed at the onset of the experiment; lanes 1 and 3, one week after removal of the adrenal or thyroid glands, compared with normal adult controls (A(N), lanes 4, 5). In adult hypophysectomized (A(H)) animals there is a significant reduction in CREM τ one week after treatment (lanes 2, 7), and after 2 weeks (lane 8). There is no CREM switch in hypophysectomized prepubertal (P(H)) animals after 1 week (lanes 12, 13) (20–23 days old; compare lanes 12 and 13 with lanes 9–11, P (H and P(N)). Each lane was loaded with an equivalent amount of RNA (see G6PD reverse-transcribed PCR controls: **a**, lanes 1–9 and **b**, lanes 1–3, and α -actin hybridization of the CREM northern blot, **b**, lanes 4–13).

METHODS. Operated rats were obtained from the Centre de Service des Animaux de Laboratoire, CNRS, Orléans. RNA was analysed as described¹. The sequences of PCR primers were: for *Hox-1.4* (5'), 5'-ATGAAGAAGATCCACGTGAGCGC-3', and (3'), 5'-AGGCAGTGTGGAAGATCGCATC-3' (positions +500 to +522 and +1,233 to +1,255 in ref. 5). Southern blots of *Hox-1.4* products were hybridized with an end-labelled 35-nucleotide antisense oligonucleotide: 5'-ATTCCTTCTCCAGTCCAAGACTTGCTGCCGGTA-



TAGGC-3' (positions +1,093 to +1,054 in ref. 5). CREM PCR primers have been described (primers A and C in ref. 2). Southern blots were hybridized with the full-length CREM α cDNA probe². G6PD primers were: (5'), 5'-ATCTACCGCATGACCACTACCTG-3', and (3'), 5'-CCCACAGAAGACATCCAGGATGAG-3', which correspond to sequences within exons 6 and 11 of the rat gene. Southern blots were hybridized with a cDNA antisense oligonucleotide probe: 5'-TTTCATCCTGCGCTGTGGCAAAGCTCTGAATGAGCGCAAAGCTGAA-3'. The migration positions of ribosomal RNA 18S and 28S size markers are indicated.

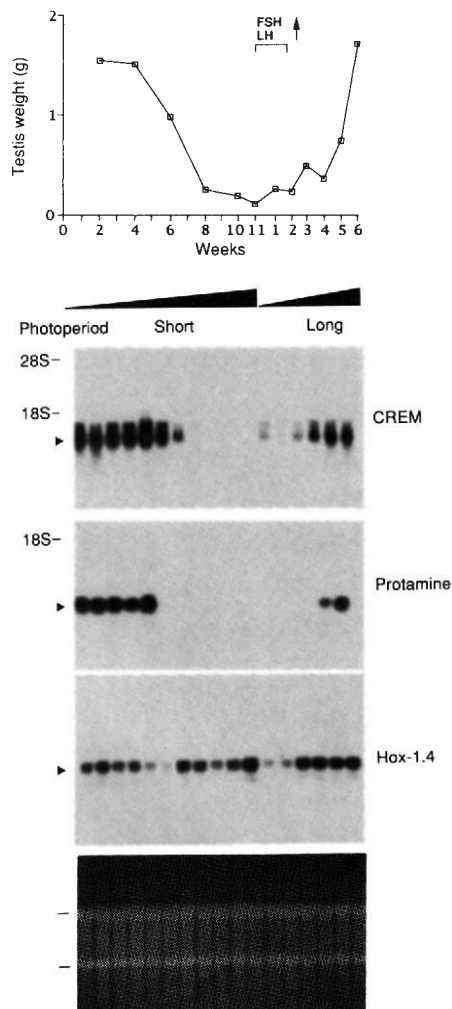
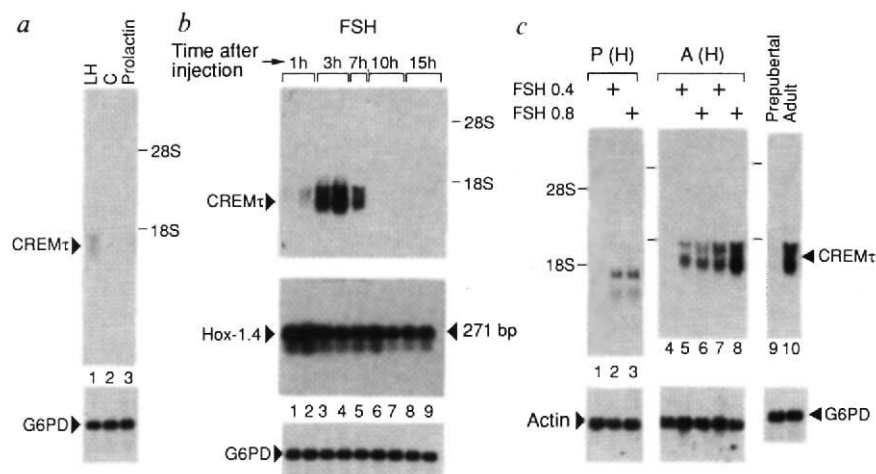


FIG. 2 Photoperiod control of CREM expression in hamster testis. Golden hamsters were exposed initially to short photoperiods (SP) for up to 12 weeks and then switched to long-photoperiod (LP) conditions for up to 6 weeks. Top panel, the weight of the dissected testis was determined and plotted against time. The period during which FSH and LH are induced following the change to long photoperiod conditions is indicated by a bracket⁴. Middle panel, total RNA prepared from each of the animals was analysed by northern blotting using CREM and protamine probes^{1,16}. Bands corresponding to these transcripts are indicated. *Hox-1.4* expression was also tested by Southern blotting of RNA-PCR reactions. CREM expression is dramatically reduced in the atrophied testis, whereas *Hox-1.4* expression is not significantly affected. Protamine is also not detected in the atrophied animals, but compared with CREM there is a delayed recovery of expression upon restoration of the long photoperiod. These results were reproduced using several animals in independent experiments. During the SP, for each 2-week time point, results from two animals are shown; in the LP, results from only one animal per 1-week time point is shown. Bottom panel, equivalent amounts of total RNA were analysed for each sample in a stained agarose gel used for northern analysis and by G6PD RT-PCR internal controls (not shown).

METHODS. Adult male hamsters were obtained from Harlan CPB (Zeist, The Netherlands). Animals were exposed to short (SP, 10 h light/14 h dark) or long (LP, 14 h light/10 h dark) photoperiod regimes as described¹⁷. RNA extractions and subsequent analysis have been described (ref. 1, and see Fig. 1 legend). The protamine probe consisted of a 235-bp PCR-generated cDNA fragment from the mouse *mp1* protamine gene¹⁶.

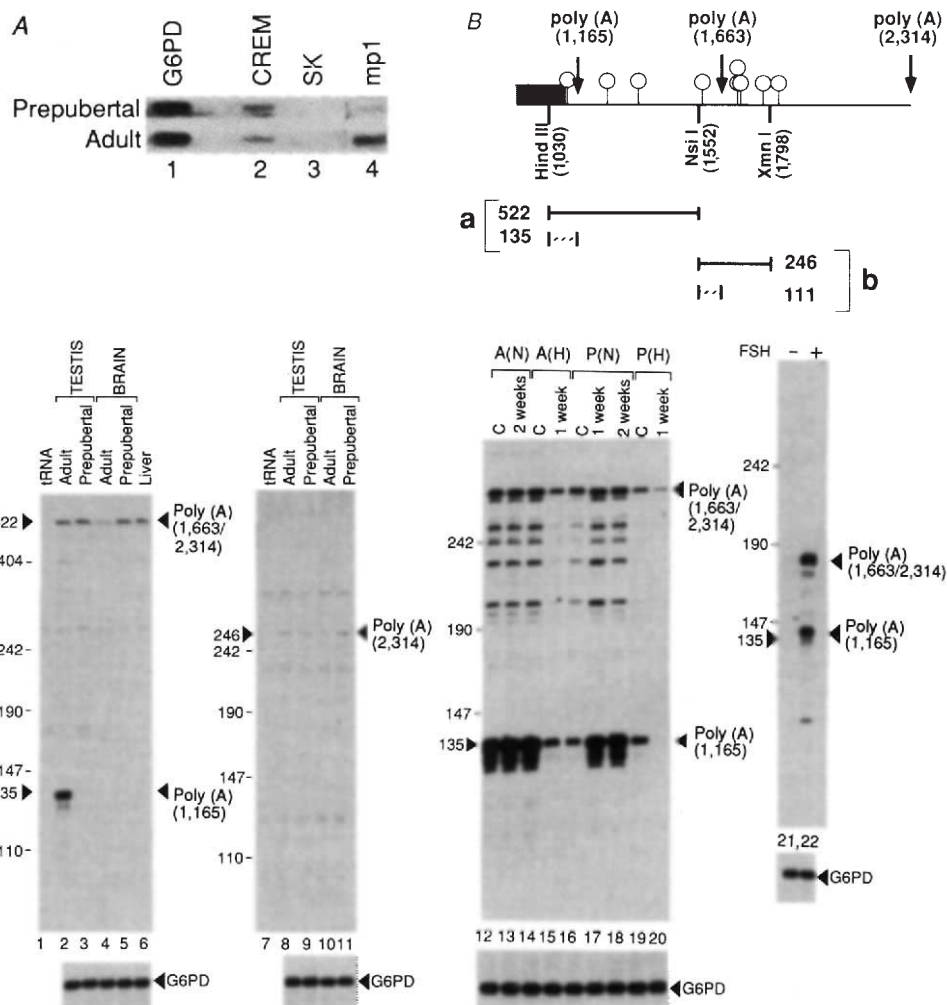
FIG. 3 FSH injection induces CREM expression in atrophied testis. **a**, Adult hamsters maintained in short photoperiod for 10 weeks were injected with doses of LH and prolactin and killed 3 h later (lanes 1–3; 'C' is uninjected control). **b**, Animals were also injected with FSH and then killed 1, 3, 7, 10 or 15 h later (lanes 1–9). Testis total RNA was analysed for CREM τ expression by northern blotting. *Hox1.4* expression was also tested using RNA PCR followed by Southern blotting (lower panel). CREM τ and *Hox-1.4* bands are indicated. There is a significant rise in CREM τ expression 3 h after FSH injection (lanes 3 and 4) to a level equivalent to that in animals maintained in long photoperiod (compare with Fig. 2). After 7 h, the level of CREM transcript decreases to non-injected control levels (lanes 6–9, and compare with lane 2 and Fig. 2). Analysis of *Hox-1.4* expression by Southern blotting of RNA-PCR products demonstrated that there is no modulation of *Hox-1.4* expression during the treatments (lower panel, and data not shown). **c**, Adult (A) and prepubertal (P; 20–23 days old) rats were injected with 0.4 IU or 0.8 IU FSH one week after hypophysectomy and then killed 3 h later. Non-injected animals (lanes 1, 4, 9 and 10) and a normal non-operated animal (lanes 9 and 10) are included as controls. Each lane of FSH-injected animals represents a single individual, confirming reproducibility. Equal loading and quality of RNAs was confirmed by G6PD RT-PCR controls or by α -actin northern analysis



(lanes 1–8). These results have been reproduced in several independent experiments.

METHODS. Adult and prepubertal animals were injected subcutaneously with 0.4 or 0.8 IU per injection of FSH (ovine), 3.5 IU/per injection of LH (human pituitary) or 0.5 IU per injection of prolactin (sheep) (all from Sigma). RNA analysis as in Fig. 1 legend.

FIG. 4 **A**, CREM expression switch is not associated with increased transcription. Nuclear run-on analysis with prepubertal (12 days old) and adult mice testes. Duplicate slot blots carried the CREM α cDNA clone (CREM) and pBluescript (SK) (3 μ g of each plasmid) together with a 755-bp PCR-generated fragment of the hamster glucose 6-phosphate dehydrogenase cDNA (G6PD) (3 μ g) and a 235-bp PCR-generated fragment (3 μ g) of the mouse *mp1* protamine cDNA (see Fig. 2, and ref. 16). There is no difference in the transcription rate of CREM between adult and prepubertal testis. The transcription rate of G6PD¹⁸ is also constant. The protamine *mp1* gene transcription is significantly increased¹⁶. **B**, The abundant CREM τ testis transcript is polyadenylated at an alternative site which confers increased stability. Top, schematic representation of the 3' untranslated region of CREM. The three polyadenylation signals (polyA) indicated are non-canonical¹; each AUUUA destabilizer is represented by a circle. The extent of the two RNase-protection probes (a and b) and the delimiting restriction sites are indicated¹. Positions of the polyadenylation sites with respect to the mouse CREM α cDNA sequence¹ are indicated in parentheses. Bottom panels: lanes 1–11, RNase protection analysis with 10 μ g RNA from mouse adult and prepubertal testis, adult and prepubertal brain and liver, using probes a (lanes 1–6) and b (lanes 7–11). Specific protected bands (see **B**, top panel) are indicated. Lanes 12–20, equivalent analysis of testis RNA from normal and operated rats using probe a (see also Fig. 1b). Lanes 21, 22: RNA from atrophied hamster testis treated (lane 22) or not treated (lane 21) with FSH using probe a (see Fig. 3b, lane 3). Bands arising from the use of specific polyadenylation sites are labelled with the equivalent position of those sites in the mouse gene. Equivalent loading was confirmed by G6PD reverse-transcribed PCR internal controls (see also α -actin control, Fig. 1b, lanes 4–13). The size of the fully protected probe (corresponding to 522 bp in the schematic diagram) is reduced owing to mismatches with the rat and



hamster CREM transcripts (unpublished results).

METHODS. Nuclear run-on and RNase protection analyses were done as described¹⁹. Probes a and b are 522-bp *HindIII*-*NsiI* and 246-bp *NsiI*-*XmnI* pBluescript subclones, respectively, from the 3' untranslated region of CREM α (ref. 1).

unaffected by hypophysectomy, indicating that it is not under direct pituitary control (results not shown; Fig. 2).

We better defined the role of pituitary hormones using a more physiological system. In golden hamsters, seasonal variation of gonadal activity is characterized by cessation of spermatogenesis during winter⁶. This modulation is dependent on the photoperiod and can be experimentally reproduced by artificial lighting⁷. Constant short photoperiod causes complete gonadal atrophy within eight weeks^{6,8}. Atrophy is accompanied by a reduction in testosterone and pituitary hormone production^{9,10}. Restoration of long photoperiod causes a sharp rise in FSH, luteinizing hormone (LH) and prolactin levels, and then a progressive increase in testis size and recovery of spermatogenesis^{4,9}.

There is an important reduction in CREM expression during the short photoperiod-induced gonadal atrophy (Fig. 2). Upon restoration of long-photoperiod CREM τ expression increases. The absence of CREM τ expression in the atrophied testis coincides with low levels of circulating gonadotrophic hormones (data available on request from the authors). Atrophied testes still contain spermatocytes and some early spermatids⁷, however, implying that CREM expression is not solely under developmental control. *Hox-1.4* expression, although variable, persists through long and short photoperiods (Fig. 2). This confirms that *Hox-1.4*, unlike CREM, is not under direct pituitary control. Expression of the 'housekeeping' gene glucose-6-phosphate dehydrogenase also appeared to be constant. These data further implicate gonadotrophic hormones as effectors of CREM expression in testis.

To demonstrate the function of pituitary hormones directly, we injected FSH, LH and prolactin into adult hamsters with atrophied testis. There was no detectable increase in CREM expression with LH and prolactin (Fig. 3a, lanes 1–3). In contrast, FSH injections led to a rapid and significant increase in the amount of CREM τ transcript, which peaked within three hours and returned to basal levels after seven more hours (Fig. 3b). CREM τ expression in FSH-induced animals was equivalent to that in sexually active adult animals (lanes 3 and 4). The cellular composition of the seminiferous tubules from FSH-induced and non-induced animals was equivalent (not shown). *Hox-1.4* expression appeared to be constant in all animals, confirming its differential regulation with respect to CREM (Fig. 3b). FSH injection also activates CREM expression in the atrophied testes of hypophysectomized rats. In both prepubertal and adult surgically treated animals, injection of FSH led to the induction of CREM expression within 3 h (Fig. 3c). These findings confirm the crucial role for FSH in CREM expression in testis.

To investigate the molecular mechanism behind CREM induction, we performed nuclear run-on assays. There is no CREM transcriptional activation in adult relative to prepubertal testis, whereas protamine gene transcription is significantly increased (Fig. 4A). Also, as there are ten AUUUA mRNA destabilizer elements within the 3'-untranslated sequence of CREM τ (Fig. 4B, refs 1, 11), inducibility could be related to alteration of transcript stability. We have identified three alternative polyadenylation sites by cDNA cloning and systematic RNase-protection analysis (Fig. 4B, and refs 1, 2; data available on request). We show that the developmental switch correlates with the use of the most 5' polyadenylation site in the adult (Fig. 4B, lanes 1–11). The use of this site generates a more stable transcript because nine destabilizer elements are excluded. The switch between alternative polyadenylation sites also occurs in operated rats. Specifically, the decrease in the amount of CREM τ transcript after removal of the pituitary corresponds to the lack of transcripts polyadenylated at the most 5' site (lanes 12–20). Interestingly, a significant proportion of the FSH-induced CREM τ transcript is polyadenylated at the 5' site (lanes 21, 22). Thus, the use of the 5' polyadenylation site correlates with accumulation of the CREM transcript.

CREM is unique among the CRE/ATF (activator transcription factor) family of genes¹² because its expression is inducible and finely regulated¹³. CREM is also the first example of a gene whose expression is modulated by a pituitary hormone during spermatogenesis. Although Sertoli cells have FSH receptors, this is not obviously the case for germ cells^{14,15}. Thus, the effect on CREM expression described here could be mediated by yet another hormonal messenger whose function would be to connect Sertoli and germ cells. Indeed, intimate interactions between these cell types have been described¹⁵. One important implication of the FSH function illustrated here is that hormones can regulate gene expression at the level of RNA processing and stability. The wider consequence of these events is that post-transcriptional events can constitute important targets for intracellular signal transduction pathways. □

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Thermodynamic β -sheet propensities measured using a zinc-finger host peptide

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THE three-dimensional structures of proteins reveal that the distribution of amino acids within the major classes of secondary structure is not random but that each amino acid has its own preferred secondary structural arrangements^{1–5}. Propensity scales for residues in α -helices have been generated through the use of various host-guest systems^{6–9}. Here we measure the thermodynamic β -sheet propensities of each of the twenty commonly occurring amino acids. A previously studied zinc-finger peptide¹⁰ was used as the host system in which amino acids were substituted into a guest site, a solvent-exposed position in an antiparallel β -sheet. As these peptides are unfolded in the absence of bound metal but are folded in their presence, it is assumed that the thermodynamics of metal binding fully reflect peptide-folding

a

Pro	Tyr	Xaa	Cys	Pro	Glu	Cys	Gly	Lys	Ser	Phe	Ser	Gln
1	2	3	4	5	6	7	8	9	10	11	12	13
Lys	Ser	Asp	Leu	Val	Lys	His	Gln	Arg	Thr	His	Thr	Gly
14	15	16	17	18	19	20	21	22	23	24	25	26

b

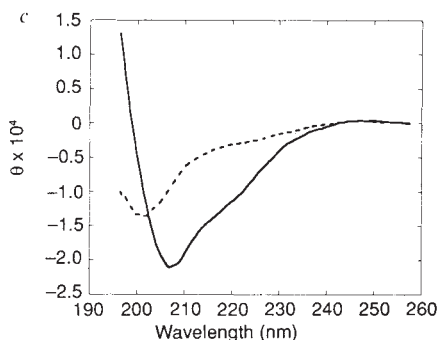
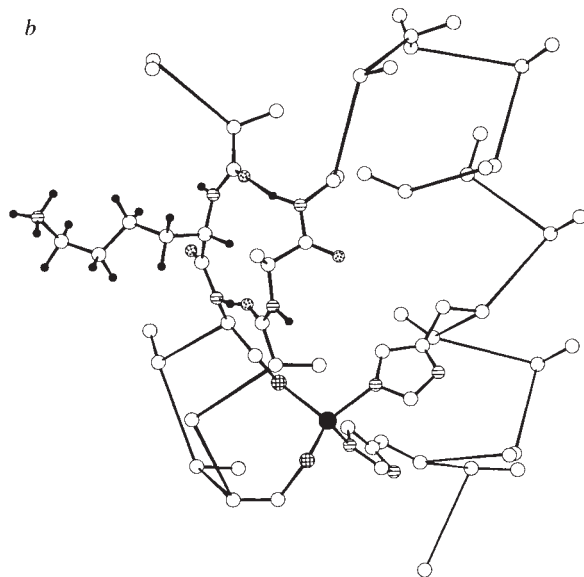


FIG. 1 a, The primary structure of the host peptide CP-1. Xaa in position 3 is the guest site. A second peptide, CP-1(CCHC)¹⁰, was used in the dual titrations together with the amino-acid variant host peptide and has the same sequence as CP-1, except that His 24 is replaced by Cys, with Lys in position 3. b, A three-dimensional structural representation of the metal-bound zinc-finger host peptide based on two-dimensional NMR studies. Structure shows the local β -sheet structure with Lys in position 3 as well as the metal-binding residues. Only α - and β -carbon positions are shown for the rest of the structure. c, Circular dichroism (CD) spectra of the Ile variant host peptide. The dashed line is the spectrum of the apo-peptide; the solid line is the same but with added cobalt(II). The apo-peptide spectrum resembles others reported for unfolded zinc-finger peptides not bound to metal^{12,13}, whereas addition of cobalt(II) produces a spectrum very similar to those of other folded zinc-finger peptide-metal complexes. The CD spectra of the Gly variant with and without cobalt(II) were nearly identical to those of the Ile variant (data not shown). Ellipticity (θ) is expressed as $\text{deg cm}^2 (\text{dmol amino acid})^{-1}$. All peptides were made on a Milligen 9050 Peptide Synthesizer using standard solid-phase techniques; they were cleaved and purified as described¹⁰, and their identity verified by plasma or laser desorption mass spectrometry. Samples were prepared for CD and their spectra recorded as described¹⁸. All manipulations with reduced purified peptide were under anaerobic conditions.

The coupling of these processes allows metal-ion affinity to be used as a measure of folding free energy. Second, a two-peptide competitive metal-binding assay has been developed¹⁵ which allows relative metal-ion-binding free energies to be determined with high precision (Fig. 2). The different absorption spectra of the cobalt(II) complexes of the host variant peptide and of the internal standard, CP-1(CCHC) (which has the final His residue replaced with Cys; Fig. 1) allows deconvolution of measured visible spectra to yield separate fractional saturation values for the two peptides. These values were used to evaluate the relative metal-binding free energy and hence that of peptide folding. All the amino-acid-variant host peptides were titrated with cobalt(II) in this manner to obtain the relative free energies as a function of the guest residue as summarized in Table 1.

energy. A competitive cobalt(II)-binding assay was used to determine these energies with high precision. The relative free energies correlate well with previously derived potential values based on statistical analysis of protein structures. We are therefore able to present a thermodynamic β -sheet propensity scale for all the commonly occurring amino acids in aqueous solution.

We have used a zinc-finger peptide¹⁰ (Fig. 1) as the host system with a guest position within a β -strand. Position three was chosen as the guest site because it lies in a relatively well defined β -strand with $\phi = -154^\circ$ and $\psi = 148^\circ$, with the carbonyl group of the preceding residue and the amide group of the succeeding residue forming hydrogen bonds across the sheet in an antiparallel fashion (V. J. Kilfoil, B. T. Amann & J.M.B., manuscript in preparation). The side chain is essentially completely solvent-exposed and in an apparently rigid region; substitution in this position is tolerated without detectable changes in structural or solubility properties¹¹.

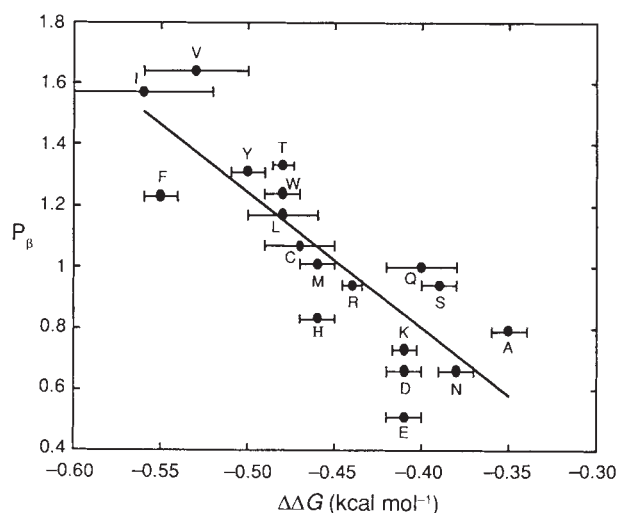
This system is suitable for a number of reasons. First, peptide folding appears to be completely coupled to metal binding for these peptides, that is, the peptides are unfolded in the absence of metal but fold into well defined structures upon binding cobalt(II) or zinc(II) (refs 12–14). This was explicitly tested for these peptides (Fig. 1c). In addition, proton NMR studies of one variant (Asn 3) confirmed that both the unfolded, apo-peptide form and the fully folded metal-bound form are monomeric and that no other species are detectable in solution.

FIG. 2 a, Plot of the fractional saturations (Y) of the Met 3 variant and the internal standard CP-1(CCHC) (here abbreviated as CCHC) and the curve fitted to their difference. b, Fitted curves for the difference in fractional saturation for Ile 3, Tyr 3, Met 3, Lys 3, Ala 3 and Gly 3 variants. For all Gly 3-variant titrations, the first 4 or 5 data points were not included when fitting the curves owing to the anomalous behaviour of this peptide at low metal concentrations. The ratio of dissociation constants, $C = K_{d1}/K_{d2} = (Y_2(1-Y_1)/(Y_1(1-Y_2)))$, was obtained from the curve fitted to the difference in fractional saturation, that is $Y_1 - Y_2 = ((1+C) - \sqrt{(x-1)^2(1-C)^2 + 4C})/(1-C)$, where $x = Y_1 + Y_2$. Subscripts 1 and 2 refer to the variant host peptide and CP-1(CCHC), respectively. $\Delta\Delta G$ values were then calculated using the equation $\Delta\Delta G = RT \ln((K_{d1}/K_{d2})/(K_{d\text{Gly3}}/K_{d2}))$. c, Typical spectra from a cobalt(II) titration. d, Deconvolution of the spectrum, which was done as described¹⁵. ●, Observed absorbance; △, calculated absorbance; ◆, Met 3; ○, CP-1(CCHC). About 200 nmol each of variant host peptide and of CP-1(CCHC) peptide solution were added to 2 ml 100 mM HEPES, 50 mM NaCl, pH 7.0. The pH of the solution was reduced to 6.8 and remained constant throughout the titration.

FIG. 3 Plot of the P_β values of Chou-Fasman (based on crystallographic data from 64 proteins⁵) versus our $\Delta\Delta G$ values. At least two titrations were done for each of the variants. Amino acid labels are in single-letter code. The average value from all titrations is plotted; error bars represent the estimated standard deviations. As a control for the assay, Ala, Val, and Gly were each substituted in positions 19 and 22, the most solvent-exposed residues on the helical region of the zinc-finger structure. The order of stability at both positions was Ala>Val>Gly (data not shown), consistent with the order derived from other α -helix host-guest studies^{8,9}. Below are listed correlation coefficients from the plots (without Pro and Gly) of our data to various other potential values. β_A and β_P refer to potentials for antiparallel and parallel β -sheets, respectively, and P_α and P_β -turn are the Chou-Fasman potentials for α -helices and turns, respectively.

Potential	Correlation coefficient with $\Delta\Delta G$
P_β (ref. 5)	0.83
P_β^* (ref. 5)	0.88
$P_\beta^\dagger$ (ref. 5)	0.84
β_A (ref. 4)	0.75
β_P (ref. 4)	0.69
β_A and β_P (ref. 4)	0.83
Ref. 3 $\ddagger$	0.80
Ref. 2 $\ddagger$	0.83
P_α (ref. 5)	0.20
P_β -turn (ref. 5)	0.54

* Correlation coefficient is derived from the plot of P_β and our $\Delta\Delta G$ values without the charged residues (Asp, Glu, His, Lys and Arg). Because parallel



β -sheets are found more often buried in the hydrophobic core of proteins¹⁹, charged residues are not frequently observed in this particular class of secondary structure. Therefore the P_β values, which are derived from the frequency of both parallel and antiparallel β -sheets, of charged residues would decrease relative to their intrinsic thermodynamic value.

$\dagger$ Correlation coefficient of P_β versus $\exp(-\Delta\Delta G/RT)$.

$\ddagger$ Values of β -potential used for plots were taken from ref. 4. Definitions and derivations for these potential values can be obtained from the references shown.

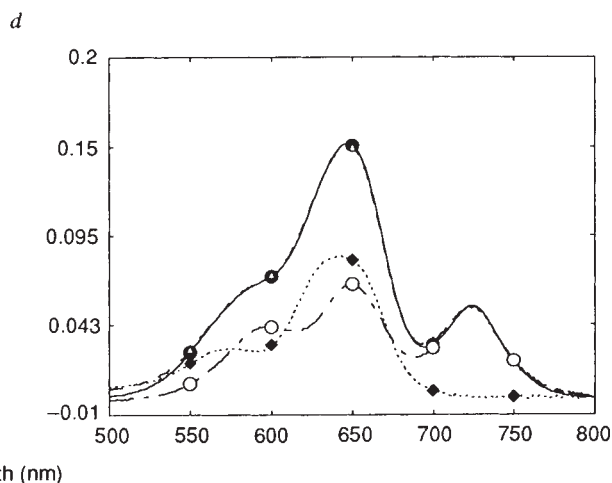
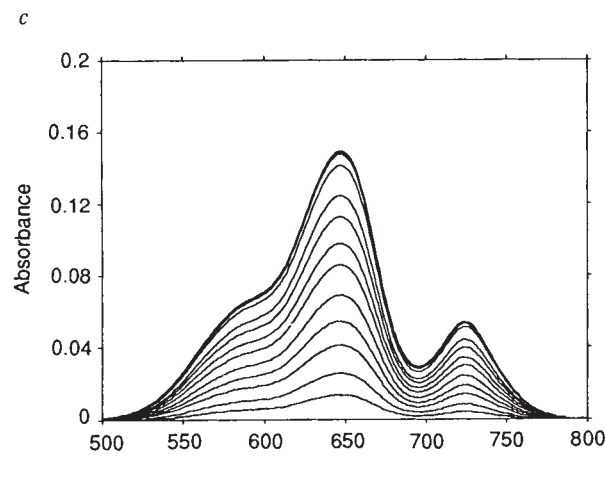
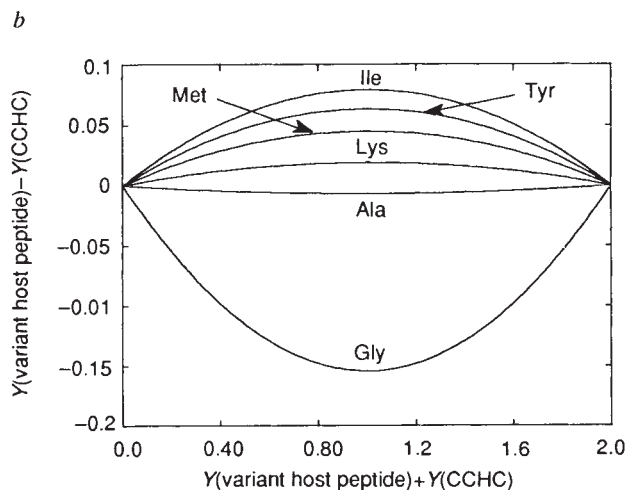
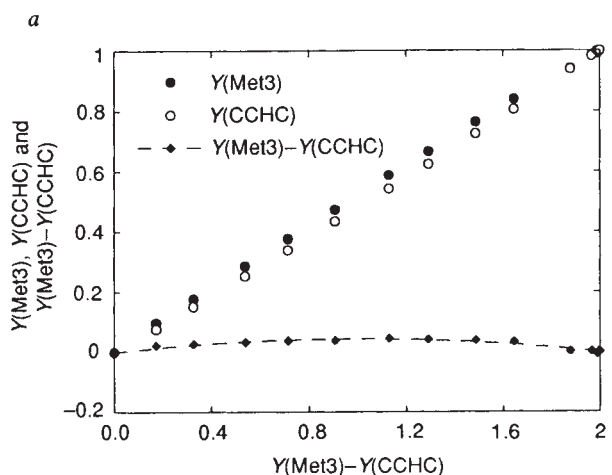


TABLE 1 Summary of β -sheet propensity data

Amino acid	$\Delta\Delta G$ (kcal mol ⁻¹)	P_{β}^*	Amino acid	$\Delta\Delta G$ (kcal mol ⁻¹)	P_{β}^*
Ile	-0.56	1.57	Arg	-0.44	0.94
Phe	-0.55	1.23	Lys	-0.41	0.73
Val	-0.53	1.64	Asp	-0.41	0.66
Tyr	-0.50	1.31	Glu	-0.41	0.51
Thr	-0.48	1.33	Gln	-0.40	1.00
Trp	-0.48	1.24	Ser	-0.39	0.94
Leu	-0.48	1.17	Asn	-0.38	0.66
Cys	-0.47	1.07	Ala	-0.35	0.79
Met	-0.46	1.01	Pro	-0.23	0.62
His	-0.46	0.83	Gly	0	0.87

*Numbers are taken from crystallographic data from 64 proteins⁵.

To assess how well these values compared with statistically derived β -sheet potential values, we plotted our $\Delta\Delta G$ values against the P_{β} values of Chou and Fasman⁵ (Fig. 3). Although the guest site in our system is on a solvent-exposed edge strand in an imperfect antiparallel β -sheet, a remarkably good relationship was observed (correlation coefficient 0.83, excluding Pro and Gly) with P_{β} , the potential value based on frequency of occurrence in all β -sheet structures. Similar plots with other β -sheet potential values⁴ yielded comparable correlation coefficients. Comparison with values for α -helix or turn potential⁵ yielded significantly lower correlation coefficients. All amino acids except for Gly and Pro were within 0.21 kcal mol⁻¹ of each other. This range is somewhat smaller than those for α -helix thermodynamic scales (0.71 kcal mol⁻¹ (ref. 8), and 0.61 or 0.42 kcal mol⁻¹ (ref. 9)). Losses of side-chain entropy can rationalize α -helix propensities¹⁶. Calculations (data not shown) based on a database of side-chain conformations as a function of secondary structure¹⁷ suggest that such effects will be more modest for β -sheets; the distributions in χ_1 angles within β -sheets resemble those for all structures, whereas those with α -helices are always more constrained.

Our data provide the first direct evidence that statistically derived β -sheet propensities have a local thermodynamic origin. The stability of different amino acids within β -strands should be tested to confirm our results and to investigate the differences between antiparallel and parallel structures and between internal and edge strands. Our scale for a solvent-exposed position in one class of β -structures shows remarkable agreement with a statistically derived potential based on all β -structures. Combined with earlier studies of α -helix and turn formation, these results should facilitate our understanding of protein structure and stability as well as protein design. □

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Supplementary information is material relevant to Articles or Letters which cannot, for lack of space, be published in full, but which is available from *Nature* on request.

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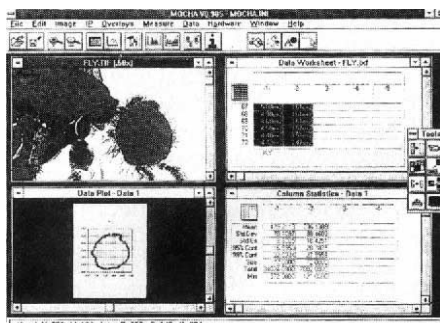
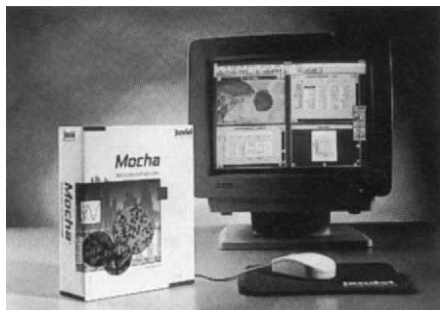
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A sharper image

An image analysis software package for Windows, a microincubator, an imaging system for autorad analysis, image processing and analysis systems and scanning electron microscopes are featured this week.

LAST month, Jandel Scientific released Mocha, a new **image analysis system** for Windows 3.1 (*Reader Service No. 100*). The program features integrated image processing and measurement, image annotation tools, a scientific data worksheet with



Mocha — Jandel Scientific's image analysis software for Windows 3.1.

transform language, and data plotting. The program runs on IBM PC and compatible computers (386 or higher) with single or dual monitors using multiple windows for display of live and stored images, worksheets, plots and statistics. Mocha supports the analysis of both colour and black and white images from frame grabbers, scanners, and disk, loading image files saved in .TIF, .BMP, .TGA, .PCX and .GIF formats. In addition, Mocha does not require a frame grabber to process and measure stored images. Researchers are, therefore, able to acquire images from core facility instruments, scanners, or off-site systems and use their own personal computers for image analysis. Jandel says that this can be of particular convenience when working with scanned images of electrophoresis gels, electron micrographs, or with images from MRI, confocal microscopes, or other instruments that save images in standard digital formats. Images can be enhanced with grey filters, image splicing, clearfield equalization and other techniques, then measured with a variety of intensity (wide line, area average and pixel intensity) and morphometric

functions (area, perimeter, major/minor axis, compactness, shape factor, and others). Mocha's integrated data worksheet (64,000 rows by 16,000 columns) offers an extensive mathematical transform language of over 50 functions, including statements for looping commands. Once analysis is complete, researchers can output images, data and plots to printers, or save image and data files in a variety of formats for use in other programs. Mocha also interacts directly with SigmaPlot for Windows for additional presentation capabilities. Mocha costs US\$3,995, but until the end of April it will be available at an introductory price of US\$2,995.

The new Axiovert 135 **TV inverted microscope** from Carl Zeiss is fitted with a TV port in the base of the instrument (*Reader Service No. 101*). This port will accommodate a highly sensitive residual light camera directly in the optical axis, only a few centimetres from the objective and the specimen. The reduced light path and the high light intensity provided by the company's ICS infinity optics should open up new possibilities in the fields of cell and tissue research. These include the detection, localization and kinetic behaviour of ions in living cells, the precise location of minute quantities of target molecules, and the display of kinetic behaviour of second messenger molecules using video-enhanced contrast and video-intensified microscopy techniques.

Designed primarily for **agglutination analysis**, such as blood grouping, the new AR-96 image analysis system is the result of the collaborative efforts of Quatro Biosystems and Sanguin International (a UK-based company that specializes in laboratory software for microplate applications) (*Reader Service No. 102*). With the AR-96, digital imaging is combined with software based on the Medusa 2000 package using algorithms developed specifically for the system. This preloaded software is designed to be simple to use and flexible, enabling

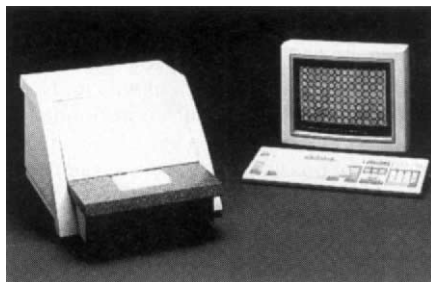
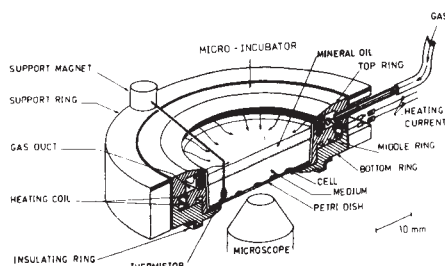


Image analysis system for blood grouping.

analysis of each microplate image to be performed according to user-defined parameters. Quatro Biosystems says that the AR-96 can read and interpret a microplate to produce a patient blood group in less than 45 s. For complete security of results, each individual microwell analysis can be overlaid on a real-time image of the microplate, which is displayed in colour on the VGA monitor. Once captured, each microplate image is stored permanently on the internal computer's hard disk for quick recall or further analysis. The AR-96 can be applied to all forms of agglutination reading, including infectious disease assays, and can be used for non-microplate applications such as gel columns.

Developed at the University of Leiden, the new LU-CB-1 **microincubator** from Medical Systems is designed to maintain cells at a constant temperature of between 30



Medical Systems' microincubator.

and 45 °C and at a constant pH on the microscope stage (*Reader Service No. 103*). A microprocessor-based temperature controller supplies the heating current. Cells are contained in a disposable 35-mm Petri dish or the reusable Leiden coverslip dish.

■ Analysis at the molecular level

The SciScan 5000 **automated imaging system** developed by United States Biochemical, which features an optical scanner and BioAnalysis software, automatically converts laboratory images into precise, consistent data for analysis and interpretation (*Reader Service No. 104*). The scanner digitally records an image of any laboratory sample with a scanning bed that accommodates various sample sizes. The computer system now features a 486DX/2 microprocessor running at 25/50 MHz, 10 MB RAM and a 16-inch full multi-synchronous monitor. New densitometry features include automatic compensation for localized background values, improved graphics capabilities, automatic analysis of fixed-format samples such as dot blots and

PRODUCT REVIEW

microtitre plates, automatic location of band boundaries for complete densitometric analysis, automatic calculation of intensities from internal standards with onscreen display, automatic calculation of molecular weight from standard markers, and qualitative reporting of normal and abnormal results from standard tests. Faster operation and more convenient editing, automatic sequence alignments, merging of multiple sequences and continuous display of the image along with merged sequences are the new DNA sequencing features.

The new PhosphorImager SF **storage phosphor imaging instrument** from Molecular Dynamics is designed to make storage phosphor autoradiography affordable to individual research laboratories (*Reader*

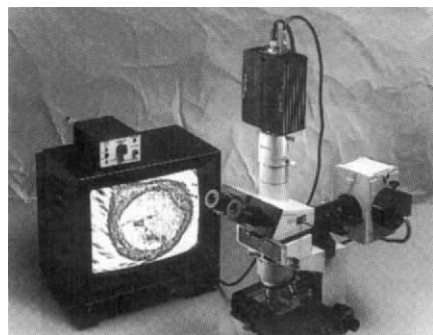


Molecular Dynamics' storage phosphor imager for autoradiography.

Service No. 105). The company says that the PhosphorImager SF accepts standard format samples (up to 20×25 cm) but is priced 30 to 40 per cent lower than Molecular Dynamics' PhosphorImager 425, which was introduced in 1989. In addition, the new system offers many of the same advantages — brief exposure times, unlimited simultaneous sample exposures, high sensitivity, and the ability to detect both strong and weak signals in one exposure.

■ Cameras/scanners

The TCC-01 from Xybion Electronic Systems is a colour **CCD camera with built-in thermoelectric cooling** that is designed to integrate an image for time periods in excess of 20 s (*Reader Service No.*



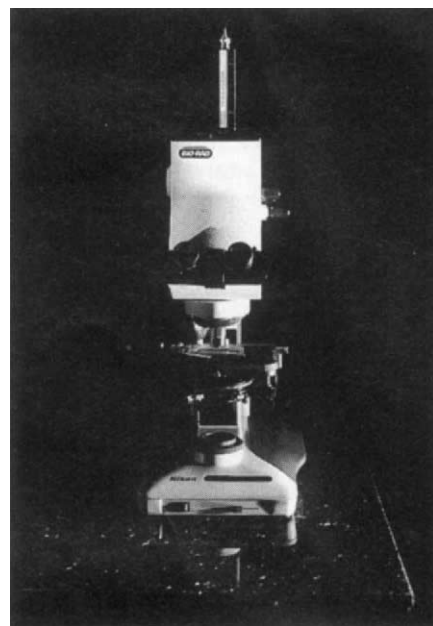
Thermoelectrically-cooled colour camera.

106). It is suitable for bioluminescence, fluorescence and other low-light-level imaging applications. The need for bulky, cumbersome cooling hoses or cable harnesses has been eliminated. A single cable between the camera and the control unit is all that is required to control power to the camera head, sensor temperature, white balance and integration time for optimum image quality and repeatability. The US\$8,350 set-up includes a camera, remote control unit and three-foot connecting cable.

In January, Nikon Electronic Imaging Products introduced Coolscan, a **film scanner** that can be used by anyone with a PC or Macintosh to input 35-mm colour and black and white negatives or transparencies (*Reader Service No. 107*). Yielding a maximum resolution of 2,700 d.p.i. and costing for under US\$2,200, Coolscan captures 24 bits per pixel in a single-pass RGB scan of both colour and black and white 35-mm negative or transparency material. The internal version, which lists for US\$2,195 and can be mounted in any 1/2 height disk drive bay, weighs only 2 lbs. For those who cannot spare a drive, the 1.2 lb US\$2,495 external version has a footprint of $5.83 \times 12.61 \times 1.85$ inches. Operation is as follows: a 35-mm slide or negative is inserted into the slot, a resolution up to 2,700 d.p.i. is selected, and Coolscan then produces an image. Nikon's patented, solid-state semiconductor light source technology features innovations in light-emitting diode fabrication and optical transmission and control. In addition to generating very little heat, the LED strobe illumination system provides fast response times (on/off cycles) and calibration stability for rich saturation and pure colours that will not change over time, Nikon says. Coolscan is initially available for Macintosh and PCs but later versions will be available for NeXT, UNIX, Silicon Graphics, SunSparcstations and IRIS workstations.

■ Confocal microscopy

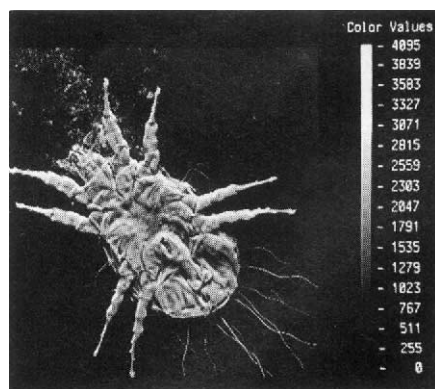
Bio-Rad says that its new ViewScan DVC-250 **confocal imaging system** provides confocal sectioning performance for everyday fluorescence applications (*Reader Service No. 108*). The system consists of a compact integrated scanhead with direct view, via the oculars, and image transference to a 35-mm camera or high-sensitivity video camera. The video camera option allows the system to be connected to computer-based image analysis systems or three-dimensional reconstruction programs. ViewScan uses a line-scanning configuration with a fixed variable confocal slit, which is positioned at the intermediate image plane of the optical system. In this configuration, the sample is illuminated by a line of light that extends across the field of view. To build an image, this line of illumination is scanned across the field of view of the microscope. Bio-Rad says that confocal line-scanning technology



Real-time colour confocal imaging system.

allows the ViewScan DVC-250 to achieve confocal imaging of the sample at high sensitivity in real time.

Meridian Instruments has introduced a dedicated confocal fluorescence model of the company's ACAS 570 interactive laser cytometer (*Reader Service No. 109*). The ACAS 570C is a stage-scanning, UV-visible **confocal system** that builds confocal images by scanning a sample over a stationary laser beam. The company says that this design, combined with a multi-lens optical element

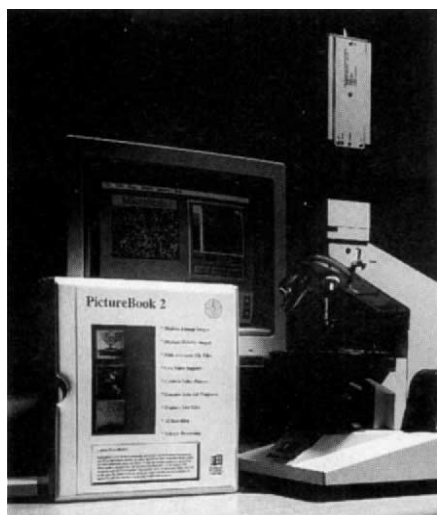


3-D ACAS 570C reconstruction of a series of confocal sections of macerated male *Tyrophagus longius* mite.

in the excitation beam path of the ACAS 570C, means that the instrument virtually eliminates both the lateral and axial chromatic aberration problems that can affect laser-scanning confocal systems. The ACAS 570C is designed to deliver consistent high-resolution imaging at all points within the field and to offer the ability to work with whole embryos or other large samples. Meridian says that it should also offer reduced photobleaching and photodamage.

■ Image processing and analysis

PictureBook 2 is a Windows-based multimedia package from Digithurst that enables the user to **collect and order information** from a wide range of sources (*Reader Service No. 110*). It also gives the user the ability to control relevant devices and invoke external software routines. Digithurst says that PictureBook is designed to provide a seamless integration between image analysis, image archiving and database handling and should be of interest to researchers seeking a bridge between analysis and archiving of photographic and video-based information. When used in conjunction with MicroScale, the company's image analysis package, it is not only possible to capture images from microscope-based cameras and endoscopes but also to position stage drivers under



PictureBook 2 — software that is designed to bridge the gap between image processing and multimedia.

software control. PictureBook's hypertext structure comes into its own in version 2.0 of the package. The user can define an object placed on a page in such a way that it acts as a gateway to a database. On selection, this object invokes Microsoft's Access database software acting on a named file. The reader may browse through the electronic book using either the hypertext links created for them by the author or their own created during searches. Pages may also contain the results of an experiment or the video feed from a camera attached to a microscope. PictureBook 2.0 costs UK£595 and requires a 386 PC with 2 MB RAM, a 40 MB hard disk, VGA display and Microsoft Windows.

The Quantimet 500 benchtop true colour **image processing and analysis system** from Leica features a single display screen that combines both menus and images (*Reader Service No. 111*). The system's QWin image analysis software is fully integrated with Microsoft Windows. Colour is used as the basis for identification and detection of objects and regions of interest. In a



True colour image processing and analysis system — see Leica.

monochrome image, Leica says that this may prove to be impossible, as entirely different colours can yield the same grey level intensity. Once objects are detected, the range of processing and measurement capabilities of the Quantimet 500 can be applied. Additionally, the colour image display allows visualization of the image where detection on the basis of grey level is deficient.

Designed for **video microscopy**, this fully integrated system from Applied Scientific Instrumentation offers computer control of two shutters, focusing and mass storage devices, including optical disk recorders (*Reader Service No. 112*). The system utilizes

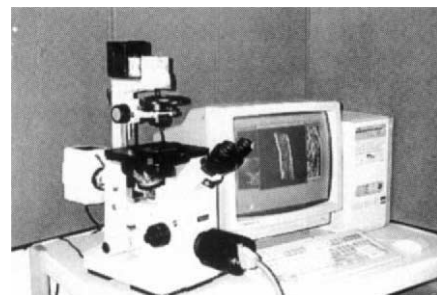


Automated time-lapse imaging system.

Axon Instruments' Axovideo software, which, the company says, provides a simple — yet powerful — image processing package. Axovideo operates on Macintosh IIs or Quadras and provides for a wide range of image processing functions. The system can be adapted to most microscopes and provides for automated, batch-mode image processing, lineage analysis, calcium imaging and all time-lapse imaging applications.

The new CELLscan **imaging system for fluorescence microscopy** from Scanalytics is designed to be a fully integrated system for the acquisition and de-blurring of images, and image analysis, providing the user with two- and three-dimensional images of fluorescently probed microscopic specimens (*Reader Service No. 113*). The processing methods used in the system allow the user to visualize and measure structures within fixed or living cells. Scanalytics says that the ability of the system's de-blurring algorithms to maintain quantitative integrity in the processed images allows the user to perform

extensive morphological and statistical measurements on the highly resolved images. The elimination of the confocal pinhole and use of a cooled CCD camera allow the system to collect all of the image data from the microscope. The company says that this



Imaging system for fluorescence microscopy.

increase in signal means that exposure times are shorter, hence reducing photobleaching and photodamage. The CELLscan system's added sensitivity makes it feasible to use lower concentrations of fluorescent probe and to use conventional light sources with standard fluorescence filter sets.

■ Scanning electron microscopy

The Orion **slow-scan grabbing board** and Elivision software package from Alrad Instruments enables a user to grab, save, retrieve, load and convert high-resolution images from an analog scanning electron microscope (SEM) (*Reader Service No. 114*). The system can be interfaced to many makes of SEM and features image averaging or integrating and electronic filtering circuits. The user selects the final image format as a function of the SEM mode (from 320×240 to $4,000 \times 4,000$ pixels with 256 grey levels).

Hitachi Scientific has launched a **scanning electron microscope (SEM)**, the S-2460 Universal, which is designed to provide high resolution at low and high vacuums (*Reader Service No. 115*). The instrument should be of particular interest in low vacuum microscopy, which is especially important in situations where samples are 'wet' with a high oil/water content. These include plant and animal specimens, which are best observed in their 'natural' habitat. The same is true for nonconducting and semiconductor materials. Hitachi says that,



Hitachi's S-2460 Universal SEM for both high and low vacuum applications.

as no complicated pretreatment of samples is required, observation is a simple procedure and no artefactual features arise from the treatment process. A sample can be introduced directly into the Hitachi Universal in much the same way as with an optical microscope. The new SEM offers a magnification of $\times 200,000$ and a guaranteed resolution of 6 nm at low vacuum. The usual range of analytical features such as elemental analysis (EDX and WDX are both available at low or high vacuums) can be added on to the S-2460 Universal. It is also designed to be a top-grade SEM at high vacuum, having a guaranteed resolution of 4 nm at 25 kV. Hitachi says that the LaB₆ option provides even higher resolution. The instrument also features two $1,024 \times 1,024$ pixel frame stores and two 512×512 pixel frame stores. Recorded images can be displayed on a four-quadrant split screen for ease of comparison. A range of image processing functions are provided to improve the quality of the image. These include a recursive filter, image integration, polarity reverse, and digitization. Images can be stored onto an optical archiving system, output on a thermal printer or recorded by photographic camera. The specimen chamber will accommodate large samples up to 150 mm in diameter and three specimen stages are available to suit individual needs.

The XL30 FEG is the latest addition to Philips Electron Optics' XL Series of scanning electron microscopes (*Reader*



XL30 FEG scanning electron microscope from Philips Analytical.

Service No. 116). The instrument offers a resolution of 1 nm at 30 kV in a conventional below-the-lens configuration and 5 nm at 1 kV via a coulomb tube. In addition to enhancing automation of the system, the XL 30 FEG's graphical user interface environment allows full integration into the laboratory information management system. The XL30 FEG's field emission system uses the same Schottky-based gun design as the company's other FEG transmission electron microscopes. Optimized both for high brightness and high beam current operation, this highly stable Schottky electron source is designed to extend considerably the application of high spatial resolution light element analysis.

The Link GEM high-purity germanium detector for scanning electron microscopy (SEM) from Oxford Instruments' Micro-analysis Group is designed to offer a guaranteed resolution of 115 eV at manganese and 65 eV at fluorene (*Reader Service No. 117*). Faster analysis, lower detection limits and improved light element analysis are features of the detector. Philips says that the Link GEM detector offers speed increases in all areas of microanalysis — element ID, quantification and X-ray mapping. Also, below 20 kV, the detector's

spectrum has no confusing escape peaks to complicate qualitative analysis. Link GEM guarantees a resolution of 133 eV at 10,000 c.p.s., compared with 1,000 c.p.s. for other systems, the company says. This tenfold increase in count means ten-times faster analysis. Although Ge detectors are not new to the industry, Philips says that in the past performance benefits have been limited to the transmission electron microscope. The Link GEM detector, however, features a new design that is optimized specifically for analysis in the SEM.

Assorted microscopes

Dyer Energy Systems, under licence from Polaroid, has introduced a **photon tunnelling microscope**, the Pegasus 1010 (*Reader Service No. 118*). Photons incident to the distal surface of an optical transducer tunnel beyond the transducer surface into an



Diamond turned acrylic lens — see Dyer.

adjacent dielectric or absorbing sample (evanescent coupling) The tunnelling probability increases exponentially with topographical height, resulting in grey-scale, visual tunnelling image. The result, the company says, is real-time, three-dimensional imaging, subnanometre vertical and enhanced lateral resolution with no probes, electrons, vacuums, dyes or physical cross-sectioning required.

Digital Instruments introduces its new manual **large sample stage scanning probe microscope**, which provides large sample capability, high resolution and ease of use (*Reader Service No. 119*). The microscope is compatible with the company's line of large sample atomic force and scanning tunnelling microscopes. It can be used to inspect the entire surface of an 8-inch diameter sample and provides 1 Å vertical resolution, Digital says. Samples are mounted on a vacuum chuck that is manually positioned under the scanning probe microscope. Tip sample engagement is automatic. The manual large sample stage is priced in the range of Digital's small sample atomic force microscopes. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

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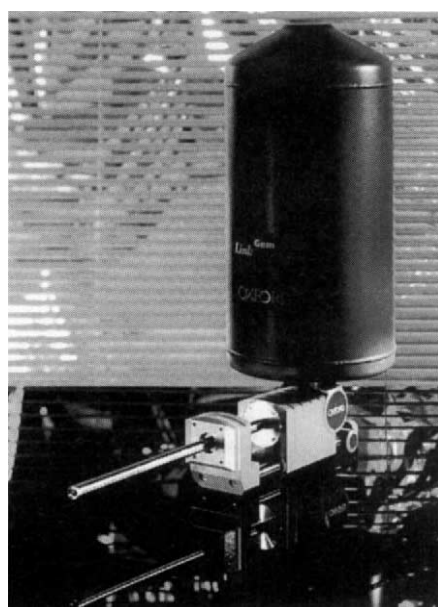
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